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SIZE, SHAPE, AND ORIENTATION OF PLOTS AND NUMBER OF REPLICATIONS REQUIRED IN SWEETPOTATO FIELD-PLOT EXPERIMENTS[1]

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INTRODUCTION

The ultimate object of field-plot experiments is not only to record the differences in behavior of plants of different kinds or of plants subjected to various treatments, but also to determine whether those differences in behavior are really significant, whether they are due to the treatments applied, or whether they are the result of some chance disturbing factor. To do this it is necessary to employ various methods for estimating the error that is certain to attend experiments of this kind. The many sources of variation that may contribute to error in results from such experiments make it essential that every available means be utilized to eliminate or reduce error in plot yields.

Numerous investigations conducted on field-plot technic during the last decade have demonstrated the importance of size, shape, and replication of plots in reducing error. The value of statistical methods in interpreting results from field experiments has become widely recognized. Those variations due to chance can be satisfactorily accounted for by statistical methods, but it must be borne in mind that errors due to poor technic cannot be removed by statistical analyses.

Salmon (*8*)[3] has pointed out some of the limitations of statistical interpretation of results from field experiments and has emphasized the importance of good judgment on the part of the experimenter. Judgment and care in selecting uniform land, in handling field operations, and in the technic used in measuring, weighing, and computing yields must be exercised, or statistical treatment of the results will be of no avail. The application of statistics to yield records will not eliminate errors resulting from careless methods in planting and in harvesting and recording yields. It is only when careful attention has been given to these operations that statistical treatment of the results can be expected to give trustworthy information. Under any other condition statistical interpretations may be misleading.

Before any extensive field experiments with a crop are made, it is important that the size of plot and the number of replications required to give accurate results be ascertained. In the spring of 1929 the experimental work outlined in this paper was begun for the purpose of determining the size and shape of plot and the number of replications

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[3] Reference is made by number (italic) to Literature Cited, p. 399.

necessary to reduce the error in plot experiments with sweetpotatoes to the lowest limit possible under field conditions.

REVIEW OF LITERATURE

A survey of the literature on the standardization of field experiments shows this to have been a fertile field for investigation during the last 20 years, as indicated by the number of articles published in scientific journals. No attempt will be made here to review the entire field; only a few articles having a direct bearing on this problem will be mentioned.

Wood and Stratton (*10*) in 1910 were among the first to apply statistical methods in interpreting results from field-plot experiments. They emphasized the need for caution in interpreting results from field experiments and outlined methods for statistical analyses. They determined the probable error of results from field plots of mangels by two methods and found it to be about 5 percent of the crop. The experimental error they found to be independent of size of plot, provided a plot of one eightieth of an acre or larger was used. By statistical analysis they determined the number of plot replications necessary to give significant reduction in error.

Mercer and Hall (*6*) in 1911 applied statistical methods to field experiments with mangels and wheat in determining the size of plot and the number of replications necessary to reduce the probable error to practicable limits. They found that the error diminished with the increase in size of the plot, but the difference in error was of little significance when the plot size was increased above one fortieth of an acre. By increasing the number of similarly treated plots and scattering them over the experimental area, they found the reduction in the probable error to be significant up to five replications, beyond which the change was insignificant. For practical purposes in field experiments they recommended the use of five plots of one fortieth of an acre each, systematically distributed over the area to be used for the experiment. They concluded that there is little difference in error between long narrow plots and square ones.

Day (*3*) in 1920, in a study of three thousand one hundred 5-foot row units of Fulcaster wheat, concluded that where plots of a given area are to be used the greatest accuracy can be obtained from long narrow plots lying in the direction of the greatest soil variation. Where the experimental area showed about equal variation in length and width, the shape of plot was found to have no influence on the results. Units of comparison composed of systematically distributed plots gave much less variable results than an equal area in a single plot.

Westover (*9*) in 1924 determined the size of plot and the number of replications necessary in field experiments with potatoes under conditions such as exist in the potato-growing districts of West Virginia. He concluded that reliable results may be obtained from the use of four systematically distributed 40-foot single-row plots.

Christidis (*2*) in 1931, in a general discussion of the question of the effect of shape of plot on the reduction of error in field experiments, presented data from various sources and made statistical comparisons between field plots of various shapes. In no instance did he find square plots to be significantly more uniform than long narrow ones.

In cases where the long narrow plots did not show an advantage over square plots, there was no indication in favor of either shape. The advantage of long narrow plots over square plots was found to be less significant when the greater dimension of the plots ran at right angles to the direction of the rows. He considered a very small factor for the ratio of width to length of plot to be desirable for yield trials from a theoretical standpoint. However, he concluded that there are some practical considerations which may make it desirable to increase the width of plot in relation to its length.

MATERIAL AND METHODS

The yield records used in the present studies were taken from four plantings of sweetpotatoes (*Ipomoea batatas* (L.) Lam.) of the variety Porto Rico, grown during the seasons of 1929, 1930, and 1931 near Beltsville, Md., and Florence, S.C. The plants were spaced 15 inches apart in rows 3 feet apart. The best plants from the first pulling from the plant bed were used; all small and inferior plants were discarded. In general, a satisfactory stand of plants was obtained from the original settings except in the 1929 planting at Beltsville. Dry weather following setting in 1929 resulted in a considerable loss of plants, but the missing hills of the entire area were replanted within 10 days of the first setting. Since weather conditions were more favorable at this time, the plants started growth more rapidly than those from the first planting, and after a few weeks there was no apparent difference in the two plantings. Little or no replanting was necessary in 1930 and 1931. The stand in all cases was about as satisfactory as could be obtained on areas as large as those used.

Throughout the growing season weeds were kept down by the usual horse cultivation and hand hoeing.

Care was exercised in harvesting to prevent the movement of the roots in the row from one 15-foot unit to another and to insure the removal of all roots from the soil. It was found necessary to remove the vines from the plants before plowing out the roots.

Records were taken for the yield for each 15-foot unit of row. The yields were recorded to the nearest quarter of a pound. All small roots were excluded from the weight records, the same standard of grading being followed as nearly as possible throughout.

PLANTING IN 1929

The first planting was made in the spring of 1929 on a piece of sandy loam containing some gravel near Beltsville, Md. The yield records were taken from an area of approximately 1 acre consisting of 50 rows 300 feet long.

The land was spring-plowed and fertilized at planting time with 600 pounds of a 2–8–10[4] fertilizer applied in the bottom of the planting ridges. The fertilizer was applied by means of a wheelbarrow type of distributor and thoroughly incorporated in the soil with a 7-tooth cultivator before the ridges were made. The ridges were thrown up at right angles to the direction of plowing.

The roots used for the growing of the plants were selected from a stock maintained by the Division of Fruit and Vegetable Crops and Diseases for a number of years and were grown near Kenilworth,

[4] Fertilizer formulas herein used refer to nitrogen, phosphorus, and potassium in the order named.

Md., in 1928. They were bedded in manure-heated plant-growing frames under glass at the Arlington Experiment Farm, Rosslyn, Va., on April 10 and 11. The "draws" were set in the field May 15 and 16. As already mentioned, dry weather prevailed at planting time, and this resulted in some loss of plants, necessitating replanting. The precipitation during the season was below normal, and a very low yield resulted. The crop was harvested October 8 and 9.

PLANTING IN 1930

The material for the 1930 study was grown on a sandy loam soil at the Pee Dee Experiment Station (a substation of the South Carolina Agricultural Experiment Station) at Florence, S.C. This land is typical of the soil used for sweetpotato production throughout Virginia and the Carolinas. The land had been in cotton in 1929 and had received an application of 840 pounds per acre of a 3–9–3 fertilizer. Previous to planting in 1930 the area used was given 600 pounds per acre of a 3–8–9 fertilizer and planting ridges were run across the direction of plowing.

Records were taken from an area of approximately 1.6 acres consisting of 130 rows 180 feet long. The plants were set in the field on May 30, the same methods being employed as had been used at Beltsville in 1929. The slips for planting were grown from roots selected from the planting stock of the Porto Rico variety, which had been used for a number of years in cooperative work with sweetpotatoes by the United States Department of Agriculture and the Pee Dee Station. The crop was given the usual care during the season. While many parts of the country suffered from drought during the season of 1930, the sweetpotato crop at the Pee Dee Station was not injured materially. It was harvested October 22 and 23 in the same manner as in 1929, and a fair yield was obtained.

PLANTINGS IN 1931

Two lots of material were grown in 1931. The first planting was made near Beltsville, Md., on the same type of soil as in 1929. Yield records were taken from an area of approximately 2 acres, consisting of 100 rows 300 feet in length. The same stock was used for plant production as in 1929. The roots were bedded in sand in water-heated frames on April 20 and 21.

The land was spring-plowed and a cover crop of rye which had been on the land during the winter was turned under. In the spring of 1930 this land had also received a green-manure crop of rye and 750 pounds of a 2–8–10 fertilizer per acre, but on account of extreme drought during the growing season of 1930, no crop had been produced. The land was prepared for the setting of the plants in the same manner as in 1929, an application of 750 pounds of a 2–8–10 fertilizer being used. The planting ridges were run across the direction in which the land was plowed.

The plants were set in the field on May 25 and 26, and the usual care was given the crop during the growing season. The crop was harvested and the yield records taken on October 8 and 9 in the same manner as in previous years.

The second planting in 1931 from which yield data were recorded comprised slightly more than 1 acre of sandy loam soil at the Pee Dee

Station. The land had received an application of 500 pounds per acre of a 4–8–4 fertilizer in the spring of 1930 and a crop of corn had been grown. Before the sweetpotatoes were planted in 1931 the area was given an application of 600 pounds of a 3–8–9 fertilizer per acre. This was the only one of the four plantings in which the rows paralleled the direction of the plowing.

The plants were set in the field on June 1, and the crop was given the usual cultivation during the season. The roots were harvested and yield records for 15-foot row units recorded November 10 and 11. The rainfall during the season was below normal, and this resulted in a low yield for land of this type in the locality of Florence.

PRESENTATION OF DATA

SIZE OF PLOT

Since four lots of material were used in making the statistical studies, it seems best to present the results for each separately and to make a final comparison of the results obtained. Each of the four lots of field data was arranged in single-row plots of various lengths and in multiple-row plots consisting of various numbers of 15-foot rows.

From the frequency distribution of yields of each size and shape of plot the mean and its probable error were computed. The standard deviation and its probable error, the error of a single determination, and the coefficient of variability were also computed.

The following formulas were employed in calculating the various measures of variability:

$$\text{Mean yield (pounds per acre)} = \frac{\Sigma\,(fV)}{n} \tag{1}$$

$$\text{Probable error of mean} = \pm\frac{0.6745\ \sigma}{\sqrt{n}} \tag{2}$$

$$\text{Standard deviation} = \sqrt{\frac{\Sigma\,(fd^2)}{n}} \tag{3}$$

$$\text{Probable error of standard deviation} = \pm\frac{0.6745\ \sigma}{\sqrt{2n}} \tag{4}$$

$$\text{Probable error of a single determination} = \pm 0.6745\ \sigma \tag{5}$$

$$\text{Coefficient of variability} = \frac{100\ \sigma}{M} \tag{6}$$

The significance of differences in standard deviation for various sizes of plots was determined by the probable error of the difference. A difference of more than three times its probable error was assumed to be significant.

In assembling the field records for single-row plots, each set of records was arranged so that the entire planting was considered as a single linear row; that is, the 15-foot units were taken consecutively down one row and back the next throughout the entire area.

In forming the multiple-row plots the adjacent 15-foot units were taken as they were arranged in the field. Since the number of field

rows in each planting was not divisible by the number of rows per plot for all plot sizes, there were some odd rows in certain cases. These were eliminated, so that all plots might be composed of adjacent parallel rows.

The number of plot replications required, as given in the last columns of tables 1 to 5, inclusive, was determined by formula 7 as explained under the discussion of plot replication (p. 392).

Experiments at Beltsville, Md., 1929

A summary of the data for single-row plots from the planting made near Beltsville in 1929 is presented in table 1. One thousand single-row units 15 feet in length were available for study. Frequencies were formed for single-row plots of 15, 30, 45, 60, 75, 90, 120, 150, and 180 feet. By increasing the size of a single-row plot from 15 feet to 180 feet the error of a single determination was reduced 515 pounds per acre, 55.7 percent of which resulted from increasing the size from 15 feet to 60 feet, 70.9 percent from increasing it from 15 feet to 75 feet, and 82.7 percent from increasing it from 15 feet to 90 feet. The relation of the curves for the actual and theoretical standard deviations is shown in figure 1.

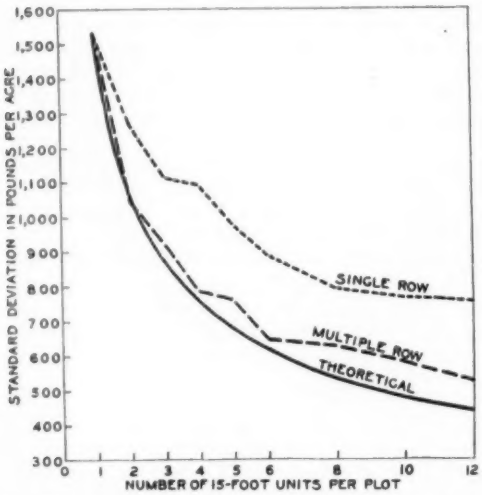

FIGURE 1.—Actual and theoretical deviations in yields for single-row and multiple-row plots of sweetpotatoes at Beltsville, Md., 1929.

The significance of differences in standard deviation for the various plot sizes is shown in figure 2, *A*. Significant differences in standard deviation were found between row lengths of 15 feet and 30 feet, 30 feet and 45 feet, 45 feet and 75 feet, and between 75 feet and 120 feet. Increasing the row length from 120 feet to 180 feet did not give a significant difference in standard deviation. No significance was found between rows of 90 feet and greater length of row up to 180 feet.

It is obvious even as a purely mathematical consideration that when a unit is replicated or increased by the amount of itself the point is soon reached where the addition of another unit has little influence on the standard deviation, since the first replication increases the whole by 100 percent, the second by 50 percent, the third by 33.33 percent, the fourth by 25 percent, and so on, each additional replication having less influence on the standard deviation. In experiments of this kind numerous varying factors enter which prevent the curve of actual standard deviations from coinciding with the theoretical curve. Where a large number of varieties or strains are to be compared, numerous

replications of each may require so large an area that the soil variation encountered may be very great. In such instances a smaller number of replications requiring less land might give a smaller standard deviation than a larger number of replications.

The one thousand 15-foot row units in the 1929 planting were arranged in multiple-row plots which included 1, 2, 3, 4, 5, 6, 8, 10,

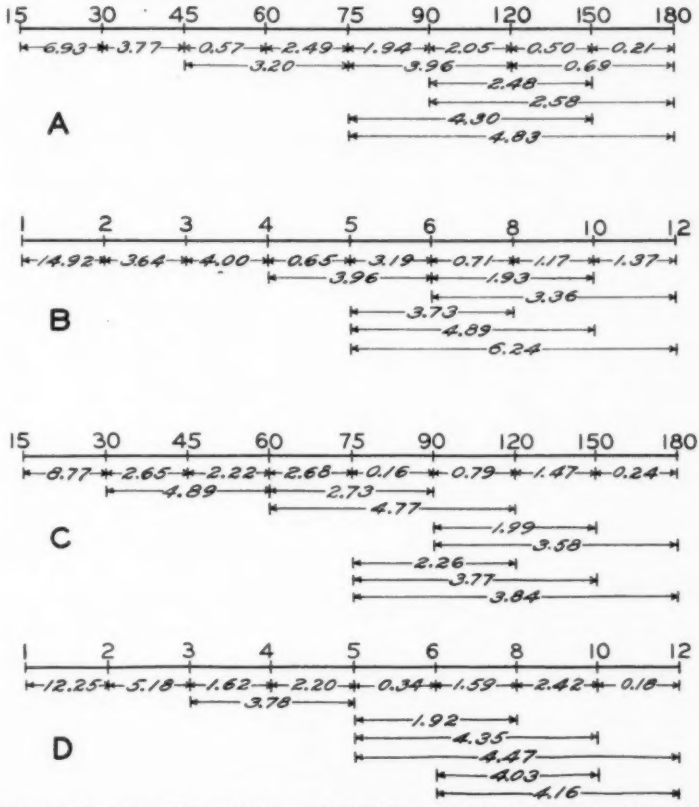

FIGURE 2.—Comparisons showing significance of reduction of standard deviation resulting from increase in size of plots of sweetpotatoes: *A*, Single-row plots, and *B*, multiple-row plots, Beltsville, Md., 1929; *C*, single-row plots, and *D*, multiple-row plots, Florence, S.C., 1930. Upper row of figures in *A* and *C* indicate length of row per single-row plot; upper row of figures in *B* and *D* indicate number of rows per multiple-row plot, with individual rows 15 feet long. Other figures represent coefficients of odds for differences between standard deviations for the plot sizes indicated.

and 12 adjacent 15-foot row units. A summary of the data for the multiple-row plots from this material is shown in table 1.

The error of a single determination was reduced 671 pounds per acre by increasing the plot size from one 15-foot row to twelve 15-foot rows. Of this reduction in error of a single determination 73.8 percent was found to be the result of increasing the number of 15-foot rows per plot from 1 to 4. Increasing the number of rows per plot to 5 produced 76.1 percent of the reduction and the 6-row plot produced 87.5 percent.

TABLE 1.—*Summary of data for single-row and multiple-row plots of sweetpotatoes at Beltsville, Md., 1929*

Number of 15-foot row units per plot	Plots		Mean yield per acre		Probable error of mean		Standard deviation		Probable error of standard deviation	
	Single row	Multiple row	Single row	Multiple row	Single row	Multiple row	Single row	Multiple row	Single row	Multiple row
	Number	*Number*	*Pounds*	*Pounds*						
1	1,000	1,000	4,687.5	4,687.5	±32.37	±32.37	1,517.5	1,517.5	±22.88	±22.88
2	500	500	4,664.0	4,660.0	±38.25	±31.42	1,268.0	1,041.5	±27.05	±22.22
3	333	320	4,641.0	4,587.5	±41.30	±34.72	1,117.5	921.0	±29.20	±24.55
4	250	240	4,668.0	4,681.0	±46.61	±34.12	1,092.5	783.5	±32.96	±24.12
5	200	200	4,667.5	4,630.0	±46.58	±36.03	976.5	760.5	±32.93	±25.65
6	166	160	4,641.5	4,681.0	±46.42	±34.53	886.5	647.5	±32.82	±24.41
8	125	120	4,646.0	4,679.0	±47.66	±38.28	790.0	621.5	±33.70	±27.06
10	100	100	4,665.0	4,655.0	±51.60	±38.88	765.0	576.5	±36.49	±27.50
12	83	80	4,614.5	4,637.5	±55.83	±39.46	754.0	523.0	±39.49	±27.88

Number of 15-foot row units per plot	Probable error of a single determination		Coefficient of variability		Plot replications necessary to meet the requirements of formula 7			
					Single row		Multiple row	
	Single row	Multiple row	Single row	Multiple row	$c=20$ percent	$c=10$ percent	$c=20$ percent	$c=10$ percent
					Number	*Number*	*Number*	*Number*
1	1,023.6	1,023.6	32.37	32.37	18.06	72.25	18.06	72.25
2	855.3	702.5	27.20	22.35	12.74	50.41	8.58	34.22
3	753.8	621.2	24.10	20.08	9.98	39.69	6.92	29.70
4	736.9	528.5	23.40	16.74	9.42	37.45	4.84	19.18
5	658.7	513.0	20.90	16.43	7.51	29.92	4.66	18.49
6	597.9	436.7	19.10	13.83	6.25	25.00	3.31	13.10
8	532.9	419.2	17.00	13.28	4.97	19.80	3.06	12.11
10	516.0	388.9	16.40	12.38	4.62	18.40	2.62	10.50
12	508.6	352.8	16.30	11.28	4.58	18.23	2.19	8.70

TABLE 2.—*Summary of data for single-row and multiple-row plots of sweetpotatoes at Florence, S.C., 1930*

Number of 15-foot row units per plot	Plots		Mean yield per acre		Probable error of mean		Standard deviation		Probable error of standard deviation	
	Single row	Multiple row	Single row	Multiple row	Single row	Multiple row	Single row	Multiple row	Single row	Multiple row
	Number	*Number*	*Pounds*	*Pounds*						
1	1,560	1,560	11,422.5	11,422.5	±39.89	±39.84	2,335.0	2,335.0	±28.80	±28.20
2	780	780	11,542.9	11,536.5	±47.14	±43.38	1,952.0	1,796.0	±33.33	±30.67
3	520	516	11,437.5	11,526.0	±51.50	±46.30	1,817.9	1,559.3	±38.02	±33.79
4	390	384	11,462.8	11,536.5	±57.85	±50.91	1,694.0	1,479.0	±40.91	±36.00
5	312	260	11,419.9	11,502.0	±58.72	±50.70	1,537.5	1,360.0	±41.51	±40.23
6	260	252	11,457.8	11,502.0	±63.90	±56.97	1,527.5	1,340.5	±45.19	±40.27
8	195	192	11,428.2	11,510.6	±67.38	±60.68	1,394.6	1,246.5	±47.60	±42.90
10	156	130	11,419.9	11,434.5	±69.88	±64.76	1,294.0	1,094.5	±49.42	±45.80
12	130	120	11,407.7	11,512.5	±74.99	±66.68	1,276.5	1,082.5	±53.70	±47.14

Number of 15-foot row units per plot	Probable error of a single determination		Coefficient of variability		Plot replications necessary to meet the requirements of formula 7			
					Single row		Multiple row	
	Single row	Multiple row	Single row	Multiple row	$c=20$ percent	$c=10$ percent	$c=20$ percent	$c=10$ percent
					Number	*Number*	*Number*	*Number*
1	1,575.8	1,575.8	20.44	20.44	7.2	28.62	7.20	28.62
2	1,316.6	1,211.4	16.91	15.57	4.94	19.62	4.20	16.70
3	1,226.1	1,051.7	15.89	13.53	4.36	17.30	3.13	12.44
4	1,142.6	997.6	14.78	12.82	3.76	14.98	2.82	11.20
5	1,037.0	917.3	13.46	11.82	3.13	12.39	2.40	9.54
6	1,030.3	904.2	13.33	11.60	3.06	12.18	2.31	9.18
8	940.6	840.8	12.20	10.83	2.56	10.18	2.02	8.03
10	872.8	738.2	11.33	9.57	2.22	8.82	1.56	6.20
12	854.9	730.2	11.11	9.40	2.15	8.47	1.51	6.00

The curves in figure 1 show the relation of the actual and theoretical standard deviation for the various single-row and multiple-row plots.

Significant differences in standard deviations for the various-sized plots are shown in figure 2, *B*. Significant differences were found between plots of 1 row and 2 rows, 2 rows and 3 rows, 3 rows and 4 rows, 4 rows and 6 rows, and between 6 rows and 12 rows.

Experiments at Florence, S.C., 1930

One thousand five hundred and sixty 15-foot single-row units were available from the planting at Florence in 1930. The data for single-row plots are summarized in table 2. Increasing the length of row per plot from 15 feet to 180 feet resulted in a reduction of the error of a single determination of 721 pounds per acre, 60.1 percent of which resulted from increasing the row length from 15 feet to 60 feet, 74.7 percent from increasing it to 75 feet, and 75.6 percent from increasing it to 90 feet.

Figure 3 shows the curves of the actual and theoretical reduction in the standard deviation resulting from increases in row length of single-row plots.

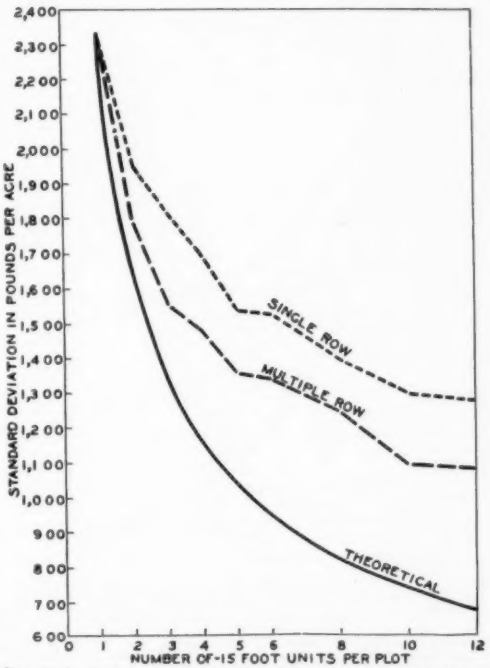

FIGURE 3.—Actual and theoretical deviations in yields for single-row and multiple-row plots of sweetpotatoes, Florence, S.C., 1930

The significance of differences in standard deviations of various row lengths is shown in figure 2, *C*. Significant differences were found between row lengths of 15 feet and 30 feet, 30 feet and 60 feet, and 60 feet and 120 feet. Increasing the row length above 120 feet gave no significant differences.

The summary of the data resulting from assembling one thousand five hundred and sixty 15-foot row units into multiple-row plots in table 2 shows the error of a single determination to have been reduced 846 pounds per acre by increasing the number of 15-foot rows per plot from 1 to 12. Increasing the number of rows to 4 produced 68.4 percent, 5 rows produced 77.9 percent, and 6 rows produced 79.4 percent of the total reduction in the error of a single determination.

The standard deviation curves are shown in figure 3.

As indicated in figure 2, *D*, significant differences in standard deviation were found between 1 row and 2 rows, 2 rows and 3 rows,

and 3 rows and 5 rows. Above 5 no significant difference in standard deviation was found until the number of 15-foot rows per plot was increased to 10.

Experiments at Beltsville, Md., 1931

The planting made near Beltsville in 1931 gave two thousand 15-foot row units. By increasing the single-row plots from 15 feet to 180 feet, the error of a single determination for single-row plots was reduced from 1,603 pounds for a 15-foot row to 886 pounds for a 180-foot row, or a reduction of 717 pounds (table 3). Increasing the row length from 15 feet to 60 feet produced 61.3 percent of the reduction; the 75-foot and 90-foot plots produced 74.2 and 82 percent, respectively, of the total reduction.

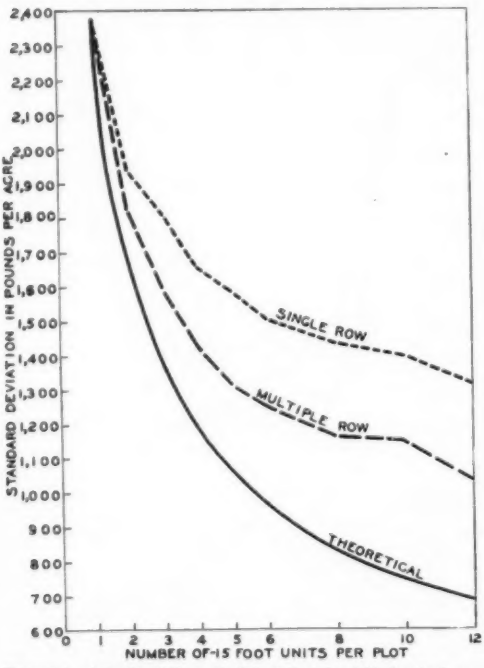

FIGURE 4.—Actual and theoretical deviations in yields for single-row and multiple-row plots of sweetpotatoes, Beltsville, Md., 1931.

Figure 4 shows the relation of the standard deviation curves.

As shown by figure 5, *A*, significant differences in standard deviation were found between row lengths of 15 feet and 30 feet, 30 feet and 60 feet, and 60 feet and 120 feet.

When this material was assembled in multiple-row plots of varying numbers of 15-foot rows, as shown in table 3, a reduction of 906 pounds per acre in the error of a single determination resulted from increasing the number of 15-foot rows per plot from 1 to 12. Of the 906 pounds, 70.5 percent resulted from increasing the number of rows from 1 to 4, 78.8 percent from increasing it from 1 to 5, and 83.8 percent from increasing it from 1 to 6.

The standard deviation curves for multiple-row plots are shown in figure 4 and the significance of differences in standard deviation in figure 5, *B*. Significant differences were found between plots of 1 row and 2 rows, 2 rows and 3 rows, 3 rows and 4 rows, and 4 rows and 6 rows. Increasing the number of rows above 6 did not give a significant difference in standard deviation until the number of rows was increased to 12.

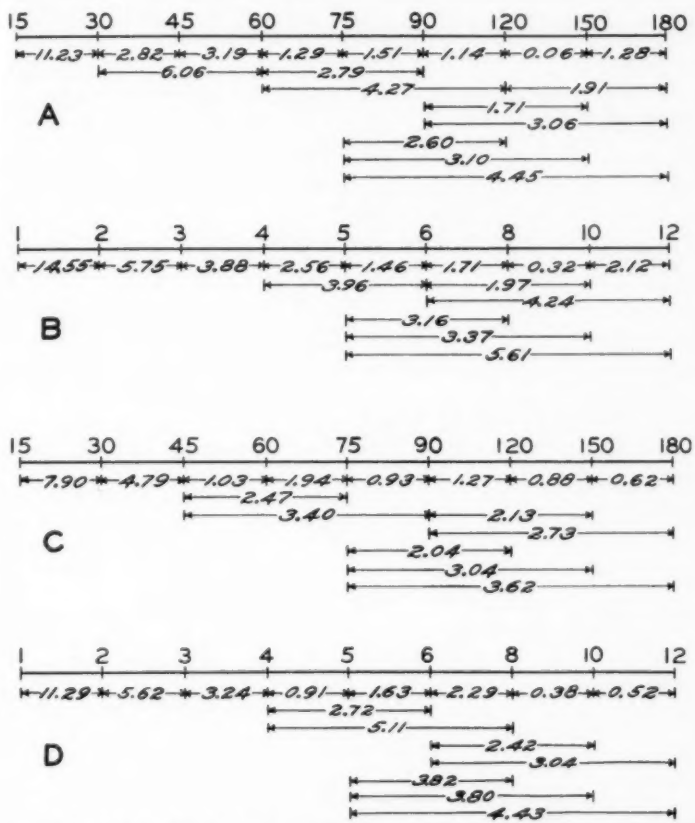

FIGURE 5.—Comparisons showing significance of reduction of standard deviation resulting from increase in size of plots of sweetpotatoes: *A*, Single-row plots, and *B*, multiple-row plots, Beltsville, Md., 1931; *C*, single-row plots, and *D*, multiple-row plots, Florence, S.C., 1931. Upper row of figures in *A* and *C* indicate length of row per single-row plot; upper row of figures in *B* and *D* indicate number of rows per multiple-row plot, with individual rows 15 feet long. Other figures represent coefficients of odds for differences between standard deviations for the plot sizes indicated.

TABLE 3.—*Summary of data for single-row and multiple-row plots of sweetpotatoes at Beltsville, Md., 1931*

Number of 15-foot row units per plot	Plots		Mean yield per acre		Probable error of mean		Standard deviation		Probable error of standard deviation	
	Single row	Multiple row	Single row	Multiple row	Single row	Multiple row	Single row	Multiple row	Single row	Multiple row
	Number	*Number*	*Pounds*	*Pounds*						
1	2,000	2,000	7,741.0	7,741.0	±35.84	±35.84	2,376.5	2,376.5	±25.75	±25.75
2	1,000	1,000	7,842.5	7,888.5	±41.36	±38.98	1,939.0	1,827.5	±29.25	±27.56
3	666	660	7,873.0	7,880.0	±47.39	±41.88	1,813.5	1,595.0	±33.52	±29.61
4	500	500	7,846.0	7,857.0	±49.92	±43.14	1,655.0	1,430.0	±36.78	±30.50
5	400	400	7,877.5	7,826.2	±53.52	±44.46	1,587.0	1,318.0	±37.84	±31.44
6	333	320	7,841.0	7,839.0	±55.60	±47.17	1,504.5	1,251.0	±39.32	±33.35
8	250	240	7,788.0	7,800.0	±61.33	±50.82	1,437.5	1,167.0	±43.36	±35.92
10	200	200	7,914.5	7,850.0	±66.52	±54.86	1,399.5	1,150.0	±47.20	±38.78
12	166	160	7,831.5	7,850.0	±68.76	±55.11	1,313.0	1,033.5	±48.61	±38.96

TABLE 3.—*Summary of data for single-row and multiple-row plots of sweetpotatoes at Beltsville, Md., 1931*—Continued

Number of 15-foot row units per plot	Probable error of a single determination		Coefficient of variability		Plot replications necessary to meet the requirements of formula 7			
					Single row		Multiple row	
	Single row	Multiple row	Single row	Multiple row	$c=20$ percent	$c=10$ percent	$c=20$ percent	$c=10$ percent
					Number	*Number*	*Number*	*Number*
1	1,603.0	1,603.0	30.70	30.70	16.24	64.48	16.24	64.48
2	1,307.9	1,232.7	24.72	23.17	10.56	41.86	9.24	36.72
3	1,223.2	1,075.8	23.00	20.24	9.15	36.24	7.07	28.09
4	1,163.0	964.5	21.10	18.20	7.68	30.47	5.71	22.66
5	1,070.4	889.0	20.14	16.84	7.00	27.77	4.88	19.45
6	1,014.8	843.8	19.19	15.96	6.35	25.20	4.41	17.45
8	969.6	787.1	18.46	14.96	5.88	23.33	3.86	15.37
10	944.0	775.7	17.68	14.65	5.38	21.44	3.70	14.75
12	885.6	697.1	16.76	13.16	4.84	19.27	2.99	11.90

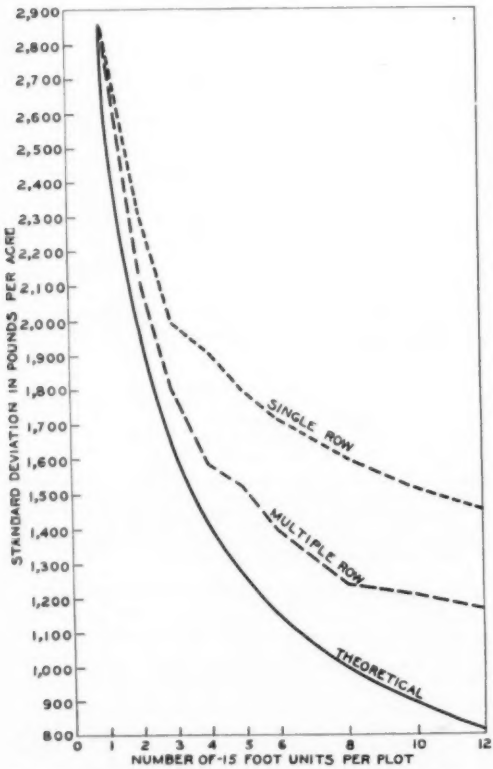

FIGURE. 6—Actual and theoretical deviations in yields for single-row and multiple-row plots of sweetpotatoes, Florence, S.C., 1931.

EXPERIMENTS AT FLORENCE, S.C., 1931

The second lot of yield records was taken in 1931 from the planting at Florence. The rows were 675 feet long. In assembling the data for the single-row plots, 23 of the 675-foot rows were used, which gave one thousand and thirty-five 15-foot units. In order to make better use of the material in assembling the multiple-row plots, one more field row was added, making one thousand and eighty 15-foot units.

The summary of data for single-row plots in table 4 shows a reduction of 951 pounds per acre in the error of a single determination as a result of increasing the row length from 15 feet to 180 feet. Increasing the row length from 15 feet to 60 feet produced 67.2 percent of the total reduction; increasing it to 75 feet produced 72.2 percent, and increasing it to 90 feet produced 81.1 percent reduction.

Figure 6 shows the standard deviation curves for the single-row and multiple-row plots.

Differences in standard deviation for various sizes of single-row plots as shown in figure 5, *C*, were found between row lengths of 15 feet and 30 feet, 30 feet and 45 feet, and 45 and 90 feet. Increasing the row length above 90 feet gave no significant differences.

TABLE 4.—*Summary of data for single-row and multiple-row plots of sweetpotatoes at Florence, S.C., 1931*

Number of 15-foot row units per plot	Plots		Mean yield per acre		Probable error of mean		Standard deviation		Probable error of standard deviation	
	Single row	Multiple row	Single row	Multiple row	Single row	Multiple row	Single row	Multiple row	Single row	Multiple row
	Number	*Number*	*Pounds*	*Pounds*						
1	1,035	1,080	8,105.0	8,089.5	±61.94	±58.02	2,862.5	2,826.5	±42.58	±41.03
2	517	540	8,138.0	8,159.0	±69.39	±62.30	2,339.5	2,146.5	±50.65	±44.06
3	345	360	8,058.0	8,125.0	±72.44	±63.73	1,994.5	1,792.5	±51.21	±45.06
4	258	270	8,112.5	8,100.0	±80.67	±65.03	1,916.0	1,584.0	±56.89	±45.97
5	207	180	8,091.5	8,055.5	±84.41	±76.39	1,800.5	1,519.5	±59.69	±54.03
6	172	180	8,032.0	8,119.5	±88.46	±70.36	1,720.0	1,400.0	±62.55	±49.78
8	129	135	8,019.5	8,059.0	±95.22	±71.98	1,603.5	1,240.0	±67.34	±49.09
10	103	90	8,102.0	8,032.0	±101.83	±86.03	1,517.5	1,210.0	±71.33	±60.83
12	86	90	8,017.5	8,088.5	±105.76	±82.91	1,453.5	1,166.0	±74.75	±58.62

Number of 15-foot row units per plot	Probable error of a single determination		Coefficient of variability		Plot replications necessary to meet the requirements of formula 7			
					Single row		Multiple row	
	Single row	Multiple row	Single row	Multiple row	$c=20$ percent	$c=10$ percent	$c=20$ percent	$c=10$ percent
					Number	*Number*	*Number*	*Number*
1	1,930.8	1,906.5	35.32	34.94	21.52	85.56	21.07	83.72
2	1,578.0	1,447.8	28.75	26.31	14.21	56.70	11.90	47.47
3	1,345.3	1,209.0	24.75	22.06	10.56	41.99	8.35	33.41
4	1,292.3	1,068.4	23.62	19.55	9.61	38.19	6.60	26.21
5	1,244.4	1,024.9	22.25	18.86	8.52	33.99	6.10	24.40
6	1,160.1	944.3	21.41	17.24	7.89	31.36	5.10	20.34
8	1,081.6	836.4	19.99	15.39	6.86	27.35	4.08	16.24
10	1,023.6	816.1	18.73	15.08	6.05	24.01	3.92	15.60
12	980.0	786.5	18.13	14.42	5.66	22.56	3.57	14.29

The one thousand and eighty 15-foot units used in the data as summarized in table 4 show a reduction in the error of a single determination of 1,120 pounds per acre resulting from increasing the number of 15-foot rows per plot from 1 to 12. Of the total reduction 74.8 percent was between 1 and 4 rows per plot, 78.7 percent between 1 and 5 rows per plot, and 85.9 percent between 1 and 6 rows per plot.

The curves of standard deviations are shown in figure 6. The differences in standard deviations for various sized plots as indicated in figure 5, *D*, show significance between plots of 1 row and 2 rows, 2 rows and 3 rows, 3 rows and 4 rows, and between 5 rows and 8 rows.

DETERMINATION OF NUMBER OF PLOT REPLICATIONS REQUIRED

Various mathematical formulas have been derived for calculating the number of replications, or number of samples distributed at random, that must be used in order that a certain difference between

two averages may be considered significant. The formula derived by Mitchell and Grindley (*7*) and applied to field-plot studies, notably by Anthony and Waring (*1*) in analyzing yield records from fertilizer experiments with apple trees, was used in this study. The value for N is determined by the formula

$$N=\left[\frac{1.849\ C\sqrt{2\tfrac{1}{2}c^2}}{100\ c}\right]^2 \qquad (7),$$

in which N is the least number that can be used when a percentage difference of c in yields between samples and odds of 30 to 1 is desired with material showing a coefficient of variability of C. The value for N as calculated by the formula can only be the most probable value, since its determination is dependent upon the coefficient of variability and must vary with the probable error of C.

The values for N as determined by the foregoing formula for the various sizes of single-row and multiple-row plots are given as the last four columns in tables 1 to 4, inclusive. The N values for the various plot shapes are given as the last items in table 5. The required number of replications has been determined for 10 and 20 percent difference for the value c in the formula.

TABLE 5.—*Summary of data on shape of plots of sweetpotatoes at Beltsville, Md., 1929 and 1931, and at Florence, S.C., 1930 and 1931*

Location and year	Rows per plot		Number of plots	Mean yield (pounds per acre)	Probable error of mean	Standard deviation	Probable error of standard deviation	Probable error of a single determination	Coefficient of variability	Number of plot replications necessary to meet the requirements of formula 7	
	Number	Length (feet)								$c=20$ percent	$c=10$ percent
Beltsville, Md., 1929	1	90	166	4,641.5	±46.42	886.5	±32.82	597.9	19.1	6.25	25.00
	2	45	166	4,671.5	±48.07	918.0	±33.98	619.2	19.65	6.66	26.42
	3	30	160	4,656.0	±45.82	859.3	±32.40	579.6	18.43	5.86	23.23
	6	15	160	4,681.0	±34.53	647.5	±24.41	436.7	13.83	3.31	13.10
Florence, S.C., 1930	1	90	260	11,457.0	±63.90	1,527.5	±45.19	1,030.3	13.33	3.06	12.18
	2	45	260	11,500.0	±61.91	1,480.6	±43.80	998.63	12.87	2.86	11.36
	3	30	258	11,469.0	±56.61	1,347.9	±40.03	909.2	11.75	2.37	9.48
	6	15	260	11,502.0	±56.97	1,340.5	±40.27	904.8	11.60	2.31	9.18
Beltsville, Md., 1931	1	90	333	7,841.0	±55.60	1,504.5	±39.32	1,014.8	19.19	6.35	25.20
	2	45	333	7,837.8	±52.45	1,419.2	±37.10	957.2	18.11	5.62	22.47
	3	30	330	7,860.5	±51.75	1,393.8	±36.59	940.1	17.73	5.42	21.53
	6	15	320	7,839.0	±47.17	1,251.0	±33.35	843.8	15.96	4.41	17.47
Florence, S.C., 1931	1	90	172	8,032.0	±88.46	1,720.0	±62.55	1,160.1	21.41	7.89	31.36
	2	45	180	8,077.8	±84.32	1,677.3	±59.63	1,131.3	20.76	7.45	29.48
	3	30	176	8,073.9	±73.27	1,441.1	±51.81	972.0	17.85	5.47	21.81
	6	15	180	8,119.5	±70.36	1,400.0	±49.78	944.3	17.24	5.10	20.34

INFLUENCE OF SHAPE AND ORIENTATION OF PLOTS ON VARIATION

In order to study the effect of shape of plot and its orientation in relation to the field rows on variation among plots, each of the four lots of material was arranged in four different plot shapes, each consisting of 90 linear feet of row.

The 90-foot row was chosen for two reasons: (1) It permits a satisfactory arrangement of the 15-foot units into the various shapes of plots containing the same number of linear feet of row; (2) the data

(figs. 2 and 5) indicated that little significant reduction in plot variability can be obtained by the use of a plot of more than 90 linear feet of row, hence it is desirable that the study be made within the limits of the largest plot shown to give a significant difference in standard deviation.

The four groups of equal plots consisted of one 90-foot row approximately 3 by 90 feet, plots of two 45-foot rows approximately 6 by 45

FIGURE 7.—Comparisons showing significance of standard deviations for 4 shapes of plots of sweetpotatoes: *A*, Beltsville, Md., 1929; *B*, Florence, S.C., 1930; *C*, Beltsville, 1931; *D*, Florence, 1931. Plots of equal area. Upper row of figures in each group show number and length of rows per plot.

feet, plots of three 30-foot rows approximately 9 by 30 feet, and plots of six 15-foot rows approximately 18 by 15 feet.

A summary of the data for the various-shaped plots for the four lots of material is presented in table 5.

Figure 7 shows the significance of differences in standard deviation between the various shapes of plots. Figure 8 presents a graphic comparison of the standard deviations for the various plot shapes for the four lots of data.

SOIL HETEROGENEITY AND VARIABILITY

The variability of the soil in the four pieces of land used for this work was determined in each case by Harris' method (*4, ch. 4*), in which the coefficient of correlation is used as an index of soil uniformity.

In applying the Harris method each field was divided into small ultimate units consisting of two rows 75 feet long. The yield from each unit was determined in pounds per acre. Groups were formed of four adjacent units. The correlation between the ultimate units and the groups was determined by the following formula:

$$r_{p_1 p_2} = \frac{\{[S(Cp^2) - S(p^2)]/m[n(n-1)]\} - p^2}{\sigma p^2} \qquad (8),$$

in which—

p = Average yield of all ultimate units.
n = Number of units in each group.
m = Number of groups.
$S(p^2)$ = Sum of squares of yields of ultimate units.
$S(Cp^2)$ = Sum of squares of the group yields.
σp = Standard deviation of the yields of ultimate units.

The constant obtained is used as a measure of the correlation between adjacent plots when grouped in a particular way.

If certain adjacent units tend to yield high and others low, a large coefficient is obtained, indicating that the field is "patchy"; a small coefficient indicates that the variation is largely due to random sampling. If the variation is due only to random sampling, the correspondence between certain contiguous units will be balanced by a lack of correspondence between others, provided the number of units is large enough to permit the expression of the law of averages.

The values in the formula for each of the four pieces of land employed in these studies are given in table 6.

TABLE 6.—*Summary of data on soil heterogeneity for the 4 areas of land used in the sweetpotato-plot studies*

[Size of ultimate units, two 75-foot rows; 4 units in each group]

Source of data	Groups	Average yield of ultimate units per acre	Sum of squares of ultimate units	Sum of squares of group yields	Standard deviation of yields of ultimate units	Coefficient of of correlation
	Number	*Pounds*				
Beltsville, Md., 1929	25	4,643.6	2,212,797,177	8,730,059,616	635.5	0.4000±0.0566
Florence, S.C., 1930	39	11,428.0	20,618,285,000	82,125,080,000	1,239.6	.5372± .0384
Beltsville, Md., 1931	50	7,833.0	12,611,195,000	49,780,610,000	1,304.0	.3488± .0419
Florence, S.C., 1931	27	8,051.2	7,222,585,625	28,477,223,525	1,420.0	.3863± .0552

The area used at Florence in 1930 was found to be the most variable, the coefficient being 0.5372 ± 0.0384. The most uniform of the four pieces was that used at Beltsville in 1931, the value for r being 0.3488 ± 0.0419. The areas used at Beltsville in 1929 and at Florence in 1931 gave correlation coefficients of 0.4000 ± 0.0566 and 0.3863 ± 0.0552, respectively.

The sources of variation other than soil heterogeneity which may influence the results of an experiment of this kind are numerous.

Where more than one thousand 15-foot units are dug, sorted, and weighed, the possibility for errors is much greater than would be the case in an actual experiment if the size of plot and number of replications recommended were followed.

Even with the best of methods it is not possible to run a large number of rows that will be exactly the same distance apart throughout their entire length; in some cases a difference of a few inches may be important. Where the plots are long and narrow and parallel the direction of the rows it is particularly important that the distances between rows be uniform.

There is always some variation in the size and vigor of plants from different parts of the plant-growing beds, especially when the heat is supplied by animal manure. Manure is quite variable in composition and heating qualities. Even in water-heated frames there may be considerable difference in growth resulting from variations in temperature. If the beds are long, the part of the frame that first receives the water from the boiler may be several degrees warmer than the end farthest from the boiler.

Plants are generally pulled and handled in such a way that a large part of one row may be set with the better plants while the next may be set with smaller and less vigorous ones. Where several setters do the work, the variations resulting from methods of setting may be considerable. Rain at the time the plants are being set may also cause variation. The amount of variation from this and other sources in field tests is difficult to determine.

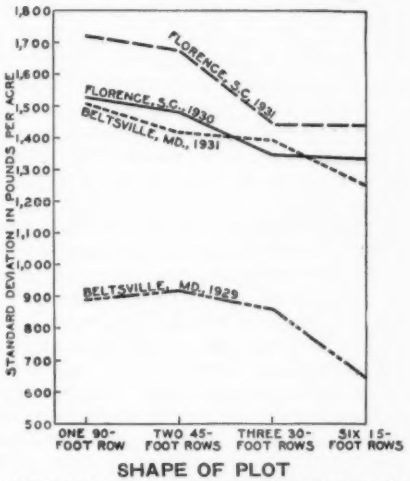

FIGURE 8.—Comparison of standard deviation curves for sweetpotato plots of equal area and varying shape.

It should be noted that the variations in plot yields for the various areas of land as indicated by the coefficients of variation given in tables 1 to 4 do not coincide with the variations in soil indicated by the coefficients of correlation in table 6; that is, the greatest variation in plot yields as indicated by the coefficients of variation did not occur on the land shown by the Harris method to be the most heterogeneous. The most heterogeneous piece of land—that used at Florence, in 1930, with a coefficient of correlation of 0.5372 ± 0.0384 (table 6)—gave the most uniform yields, as indicated by the coefficients of variation in tables 2 and 3. The land used at Florence in 1931 ranked second in soil uniformity, as indicated by the coefficient of correlation, table 6, but gave the most variable yields, as indicated by the coefficients of variation in tables 4 and 5.

The lack of correlation between soil heterogeneity and variation in yields indicates that some of the other factors mentioned contributed

materially to the variation in yields between plots. Since insufficient records were made as to the location of groups of large and small plants, the portions of rows planted by the several setters, and the variations in width of spacing between rows, it was not possible to determine the relative importance of these factors.

The greater uniformity of yields from multiple-row plots as compared with yields from single-row plots is evidence that variation existed between adjacent rows. Since it is not likely that the rows were run parallel to soil gradients in every case, and in 3 of the 4 fields the rows were run across the direction of the plowing so that variation due to dead furrows was minimized in these 3 fields, it is evident that much of the variation between rows was due to factors other than soil heterogeneity.

DISCUSSION

SIZE OF PLOT

An analysis of the data in figures 2 and 5 reveals the fact that in only one of the eight studies presented was there a significant difference in standard deviation between the various sizes of plots obtained by increasing the size of plot above 90 linear feet of row until the plot contained 180 linear feet. In the multiple-row plots of the 1930 material a significant difference was found between the 90-foot and the 150-foot plots. In every case significant differences in standard deviation were noted between plots containing 75 linear feet of row and those containing 180 feet of row. In 4 of these, significant differences were found between plots of 75 and 120 linear feet of row; in the other 4, significant differences were found between plots of 75 and 150 feet of row.

It is concluded from these data that a significant reduction in variation may result from increasing the size of plot until it includes about 90 linear feet of row. Further increase in size of the area would be of doubtful value in reducing variability between plots in field experiments with sweetpotatoes.

SHAPE AND ORIENTATION OF PLOT

The data presented in figure 7 show a significant difference in standard deviation between the 90-foot single-row plots and the plots of six 15-foot adjacent rows, in all four cases in favor of the latter. Between the other plots there was no consistent difference. The effect of row grouping in reducing variation is shown graphically in figures 1, 3, 4, and 6.

While regression curves for soil variation were not calculated and the direction of the greatest soil variation was not determined, it is not likely that in every case the rows were run parallel to such soil gradients as might have been present. Yet there was a significant reduction in the standard deviation in each case between long narrow single-row plots and almost square multiple-row plots. It seems safe to assume that much of the reduction in variation in favor of the square plots was due to the number of rows included within the plots, indicating that variation existed between rows and was probably due to factors other than soil heterogeneity.

If an appreciable amount of the variation among plots is due to correlated variations between different rows, it is obvious that plots which include portions of several rows are likely to show less variation than plots consisting of a single row. Variation between rows

resulting from methods used by plant setters can be controlled to better advantage in actual field-plot tests where the plots are set as units than where the entire area is planted as a unit, as was practiced throughout this experiment. By having each setter plant an equal portion of each plot, much of the variation from this source can be eliminated. Variation due to difference in spacing of rows can be minimized by the careful laying out of rows but cannot be entirely avoided.

Previous experiments on methods of eliminating variation in field-plot tests in most cases have indicated that there is little difference in favor of either square or long narrow plots. Where a difference has been found, it has been in favor of the latter. However, Day (*3*) points out that such plots are superior to square ones only when the longest dimension parallels the direction of greatest soil variation. Much of the work on the shape of plots has been conducted with small grains or other crops that are planted by drill, thereby eliminating some of the sources of variation that enter into transplanted crops such as the sweetpotato.

From the results of the four lots of material studied in this experiment it is concluded that square plots are superior to long narrow ones for sweetpotato field-plot tests, unless such plots are run across the field rows so as to include portions of several rows. Long narrow plots would not be the most economical of land where border rows are necessary, since the perimeter is much greater than that of square plots.

REPLICATION

The large number of replications required where significance is desired in a percentage difference of less than 20 indicates that it would not be safe to attach importance to differences of much less than 20 percent for a single year's data in field-plot tests with sweetpotatoes where any large number of treatments are to be compared. When investigations are conducted over a period of years the accumulated number of replications may be sufficient for the determination of significance of differences as small as 10 percent. The data must, however, be treated by Student's (*5*) or some other recognized method to remove correlation attributable to seasonal conditions where the results obtained in consecutive years are to be compared.

The number of replications required to give a desired degree of accuracy decreases as the size of the plot is increased within the limits stated in the study. Since analyses of the data in figures 2 and 5 show that the reduction in variation brought about by increasing the size of plot above 90 linear feet of row is of doubtful significance, it would not be economical to use plots consisting of much more than about 90 linear feet of row where the rows are spaced 3 feet apart. Even though significant reduction in variation may be obtained by the use of plots containing more than 90 linear feet of row, it is not economical of land to use the largest plot found to give significant reduction in variability.

In some cases the area of uniform land available for experimental work may be a limiting factor. The values for N as determined by the Mitchell and Grindley formula (*7*) indicate that the same degree of accuracy can be obtained by using small plots as by using large ones, with significant economy in land even though a much larger number of replications is required. For example, take the multiple-row plots of the data from the 1931 planting at Florence, which

showed the greatest variation as indicated by the coefficient of variability. By employing the formula derived by Mitchell and Grindley and using a value of 20 percent for c, the number of replications and the total linear feet of row required by the various sizes of plots are found to be, for a single 15-foot row plot, 21 replications or 315 feet of row; for a plot of two 15-foot rows, 11.9 replications or 357 feet of row; for a plot of three 15-foot rows, 8.35 replications or 376 feet of row; for a plot of five 15-foot rows, 6.1 replications or 458 feet of row; for a plot of six 15-foot rows, 5.1 replications or 459 feet of row; for a plot of eight 15-foot rows, 4 replications or 480 feet of row; for a plot of ten 15-foot rows, 3.9 replications or 585 feet of row; and for a plot of twelve 15-foot rows, 3.6 replications or 648 feet of row. Fractions of replications are used here only for the purpose of comparison. For this lot of material there is a saving in land of 9.8 percent between two and four 15-foot rows, and 11.5 percent between four and six 15-foot rows. A similar saving in land by the use of small plots is shown in all four lots of data. In some cases the saving in land is much greater. The use of very small plots has the practical disadvantage of additional expense and labor in planting and harvesting the crop; in fact the use of very small plots may result in more expense and labor than the other advantages justify. Where guard rows are necessary small plots may require even more land area than larger ones.

SUMMARY AND CONCLUSIONS

Yield records used in these studies were taken from four plantings of sweetpotatoes of the Porto Rico variety, grown in 1929, 1930, and 1931. Two lots were grown near Beltsville, Md., and two at Florence, S.C.

The field data for each planting were assembled in both single and multiple-row plots consisting of one, two, three, four, five, six, eight, ten, and twelve 15-foot row units.

The influence of plot size, shape, orientation, and number of replications on the experimental error in field experiments was determined.

Increasing the size of individual units gave a significant reduction in standard deviation of plots up to about 90 linear feet of row. Increasing the area to more than 90 linear feet of row gave an insignificant reduction in variation between plots as indicated by the standard deviations. Multiple-row plots were less variable than single-row plots of the same total row length and area.

Shape-of-plot studies were made within the limits of the maximum plot size found to give significant reduction in variation, that is, within the limits of 90 linear feet of row. Four shapes of plot were considered, consisting of areas approximately 18 by 15 feet containing 6 parallel rows 15 feet long, 9 by 30 feet containing 3 parallel rows 30 feet long, 6 by 45 feet containing 2 parallel rows 45 feet long, and 3 by 90 feet containing 1 row 90 feet long. With the exception of the 18- by 15-foot plot, the greater dimension paralleled the direction of the rows. The data as recorded in 15-foot row units were not adapted to the arrangement of long narrow plots running across the direction of the rows.

Variation among plots decreased as the plot shape approached a square. The 18- by 15-foot areas gave a very significant reduction in variation as compared with the single-row plots 3 by 90 feet.

The study of shape indicates the influence of plot orientation on variation. Variation decreased as the plot dimension at right angles to the direction of the rows was increased. Long narrow plots running across the direction of the rows probably would have given still less variation than was obtained with the plot arrangements used.

The number of replications of the various-sized plots required to obtain significance of a stated percentage difference varied with the different lots of material. Although small plots required a much greater number of replications to secure a given degree of accuracy than did large plots, there was a significant saving in land when small units were employed. The use of small areas has the disadvantage of greater expense and labor in planting and harvesting.

It does not seem advisable to suggest that any one plot size would be the most desirable in sweetpotato field experiments. It is obvious that equally satisfactory results may be obtained from plots of different sizes provided they are replicated a sufficient number of times to meet the statistical requirements. Under the conditions of a specific experiment circumstances might arise to modify the plot technic that otherwise would be the most satisfactory.

A plot consisting of approximately 90 linear feet of row replicated 4 or 5 times should give a satisfactory plot arrangement for field experiments with sweetpotatoes. The data presented indicate that a more significant reduction in variation in plot yields may be expected if the 90 feet of row is divided into a number of shorter parallel rows than where a 90-foot single-row unit is used.

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UNIFORMITY IN PATHOGENICITY AND CULTURAL BEHAVIOR AMONG STRAINS OF THE CABBAGE-YELLOWS ORGANISM[1]

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INTRODUCTION

Yellows is a disease of cabbage and of other members of the species *Brassica oleracea* L., including cauliflower, collards, brussels sprouts, kohlrabi, and kale. The causal organism (*Fusarium conglutinans* Wr.) once established in the soil remains there indefinitely as a potential parasite of these crops. Invasion of the host plant occurs in young roots, and the organism progresses upward by way of the xylem. The characteristic pathological effects have already been described (*4*).[3] As in the case of most of the diseases of herbaceous plants caused by vascular Fusaria, there is in cabbage and its allies marked variation between individuals in resistance or susceptibility to this parasite. This fact has become the basis of control through the selection of resistant strains or varieties (*2*, *3*, *5*). The early work in this direction was done by the selection of survivors from infested fields and by mass selection from standard varieties. More recent work has shown that resistance is controlled by a single dominant Mendelian factor (*1*, *4*), and the discovery of this fact was followed by selection of homozygous resistant lines from a number of varieties of cabbage.

In the program of breeding for disease resistance little attention had been given, up to the time of this investigation, to the possibility of variation in the parasite. Other workers have noted variations in the pathogenicity of vascular parasites of the genus *Fusarium* that attack other plants. It thus became highly important to the program of selection and breeding for resistance to yellows in cabbage and related subspecies that a study of the causal organism be made from the standpoint of its possible variability in pathogenicity. This paper gives the results of such a study carried on from 1927 to the present.

SCOPE OF THE INVESTIGATION

The development of cabbage strains resistant to *Fusarium conglutinans* has been carried on largely in one infested area of Wisconsin. Since specialization occurs not uncommonly among other fungi and has been reported in other vascular parasites of the genus *Fusarium*, the question of specialization was obviously of importance in breeding

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[3] Reference is made by number (italic) to Literature Cited, p. 409.

cabbage for resistance. Up to the time of this investigation little attention had been given to the question in other than a preliminary way.

The possibility of specialized races of the cabbage-yellows organism may be studied in two ways. First, lines of selected material may be grown upon infested soil in various regions and thus variations in the selective pathogenicity of the parasite may be detected. Such trials have been made in a limited way in the past, and the results have indicated that resistant selections of a number of varieties of cabbage remain resistant wherever tested. Obviously, the results of such experiments are not conclusive because of the possibility of poor infestation of the soil by the pathogene or of environmental factors that might limit full expression of the disease. A second method of determining the existence of specialized races of the pathogene is to study the organism under controlled conditions, whereby various isolates may be compared under optimum conditions that can be provided at will. The results of a study by this method are discussed herein.

METHODS AND MATERIALS

Isolations of the cabbage-yellows organism were secured from various areas of the United States where the disease occurred on cabbage or other subspecies of *Brassica oleracea* (table 1). The material was received as transfer cultures, as diseased plants, or as infested soil. In the last instance cabbage seedlings were transplanted into the infested soil and the yellows organism was isolated from the resultant diseased plants. After the recovery of the organism, monoconidial or hyphal-tip lines were made of isolations 1 to 11. Isolations 12 to 19 were not handled in this way, but were carried in culture as mass transfers from tissue plantings on agar plates. One or more hyphal-tip lines were secured subsequently from each of collections 12 to 19 and were tested for pathogenicity.

TABLE 1.—*Source of isolates of Fusarium conglutinans*

Isolation no.	Locality in which found	Type of material	Date received
1	Racine, Wis.	Infested soil	Apr. 1927
2	Ames, Iowa	do	May 1927
3	Crystal Springs, Miss.	Diseased collards plant	July 1927
4	Topeka, Kans.	Diseased cabbage plant	Do.
5	Houston, Miss.	do	Do.
6	La Fayette, Ind.	do	Do.
7	Madison, Wis.	Diseased kale plant	Do.
8	Indianapolis, Ind.	Diseased cabbage plant	Aug. 1927
9	Fayetteville, Ark.	Culture isolated from cabbage	Oct. 1927
10	Petaluma, Calif.	Culture isolated from kale	May 1928
11	Humboldt, Tenn.	Diseased cabbage plant	June 1928
12	Norfolk, Va.	do	June 1930
13	Lansing, Ill.	do	July 1930
14	Fremont, Ohio	do	Do.
15	Columbus, Ohio	do	Do.
16	Somers, Wis.	do	Do.
17	Indianapolis, Ind.	do	Do.
18	Nekoosa, Wis.	do	July 1931
19	Madison, Wis.	do	Aug. 1931

The comparative pathogenicity trials of the isolates were conducted by transplanting cabbage seedlings (or those of other subspecies of *Brassica oleracea*) into soil that had been inoculated with

cornmeal-sand cultures of the several isolates. The cornmeal-sand medium was prepared by mixing equal parts of yellow cornmeal and white quartz sand, moistening with water, and steaming for 1 hour. Approximately equal parts of the mixture were then placed in quart fruit jars, plugged with cotton, and autoclaved on 3 alternate days. Tube cultures of the isolates were used to inoculate the jars of the cornmeal-sand medium, which were incubated at room temperature for a period of 2 weeks or more. The inoculum was then mixed thoroughly with clean compost soil and placed in the metal cans of the Wisconsin soil-temperature tanks. Seedlings that had been grown in yellows-free soil for a period of 20 days or more were transplanted into the cans of artificially inoculated soil and exposed to the attack of the isolates under conditions of controlled soil temperature maintained at 22° to 24° C.

In these comparative pathogenicity studies identical conditions of inoculum and uniform conditions of soil moisture and soil temperature were maintained as closely as possible. Care was exercised at all times to eliminate the possibility of contamination of one isolate by another during the process of soil inoculation, transplanting, etc.

For the pathogenicity trials a number of varieties and strains of cabbage and other subspecies of *Brassica oleracea* were used. Homozygous susceptible lines of cabbage developed by Walker (*4*) were used, originating from several commercial varieties, as follows:

(1) HC–27–A, from a cross between All Head Early and Copenhagen Market varieties.
(2) C–29–A, from Copenhagen Market variety.
(3) SP–GC, from Late Flat Dutch variety.

Homozygous resistant lines of cabbage also developed by Walker (*4*) were used. These had been shown to remain completely resistant on infested soil up to about 24° C. Above 26° they developed disease symptoms not typical of yellows, which are discussed in detail by Walker and Smith (*6*). The following lines were used:

(1) 20–28–A and 20–30–B, developed from Jersey Wakefield variety.
(2) 30–28–A, developed from Copenhagen Market variety.
(3) 40–28–A, developed from All Head Early variety.

A number of F_2 progenies from crosses between resistant and susceptible strains of cabbage used by Walker were included. Under Wisconsin field conditions he found these to segregate close to the ratio of 3 resistant to 1 susceptible. The progenies, exact pedigrees of which are given by Walker (*4*), were as follows: 5HIA, H5ID, G20I-1s, G5IC.

In the comparative study of subspecies of *Brassica oleracea* commercial lots of seed were used, since selected lines were not available.

EXPERIMENTAL RESULTS

TRIALS WITH HOMOZYGOUS LINES OF CABBAGE

The initial pathogenicity trials of the yellows cultures were made upon seedlings of a commercial susceptible variety of cabbage. After typical symptoms of the yellows disease had been produced, the causal organism was isolated and new lots of the cornmeal-sand medium were inoculated with the cultures of proved pathogenicity. The isolates bearing accession numbers 1 to 11, inclusive, were purified by obtaining monoconidial or single hyphal-tip lines; isolates

12 to 19, inclusive, were not so handled in the early trials, although later single hyphal-tip lines were obtained and studied.

The first comparative trial of cultures 1 to 11 was made upon cabbage seedlings of 2 homozygous susceptible and 2 homozygous resistant lines. After 17 days' exposure in infested soil at a soil temperature of 24° C. the seedlings of the homozygous susceptible lines were all diseased, whereas the seedlings of the resistant lines showed no evidence of the disease. Although there was no indication of wide variation among the isolates, the number of seedlings used in the trial was too small to give great significance to the results.

In the next trial the number of seedlings was increased so that any variation in virulence might be more readily detected. The results presented in table 2 show the reaction of 1 homozygous susceptible line (HC–27–A) and 3 homozygous resistant lines (20–28–A, 30–28–A, and 40–28–A) to 10 isolates. All plants of the susceptible line succumbed to each isolate except no. 6, which induced disease in 93 percent of the seedlings. The lower pathogenicity in this case was explained by a later demonstration that a nonpathogenic contaminant was present in the inoculum jar, resulting in a dilution of the pathogene and consequently in a less virulent attack. The three resistant lines of cabbage remained entirely free from disease symptoms. The data presented in table 2 are typical of other trials, not presented here, with similar seedling material and the same cultures of the fungus.

TABLE 2.—*Pathogenicity of isolates of Fusarium conglutinans upon 3 homozygous resistant strains and 1 homozygous susceptible strain of cabbage*

Isolate no.	Plants of homozygous susceptible strain—HC–27–A		Plants of homozygous resistant strain—					
			20–28–A		30–28–A		40–28–A	
	Total	Diseased	Total	Diseased	Total	Diseased	Total	Diseased
	Number	*Percent*	*Number*	*Percent*	*Number*	*Percent*	*Number*	*Percent*
1	60	100	80	0	45	0	79	0
2	60	100	79	0	44	0	79	0
3			83	0			84	0
4	59	100	80	0	48	0	80	0
5	62	100	82	0	44	0	80	0
6	61	93	81	0	44	0	81	0
8	64	100	67	0	44	0	80	0
9	60	100	79	0	45	0	75	0
10	60	100			47	0		
11	62	100	80	0	45	0	77	0

The comparative pathogenicity trials were next extended to isolates 12 to 19, inclusive. In table 3 are presented the results of trials upon homozygous susceptible and homozygous resistant lines of cabbage. In three successive trials with a susceptible line (C–29–A) pathogenicity was uniform except in the case of isolate 16, which was less virulent than the others. Upon a second susceptible line (SP–GC) this strain eventually brought about disease symptoms in all of the seedlings, but the disease progressed much more slowly than in the case of the other isolates. The resistant lines gave no evidence of disease.

TABLE 3.—*Pathogenicity of isolates of Fusarium conglutinans upon 2 homozygous resistant and 2 homozygous susceptible strains of cabbage*

Isolate no.	Plants of homozygous susceptible strain—								Plants of homozygous resistant strain—			
	C-29-A						SP-GC		20-30-B		40-28-A	
	Trial 1		Trial 2		Trial 3							
	Total	Diseased	Total	Diseased	Total	Diseased	Total	Diseased	Total	Diseased	Total	Diseased
	Number	*Percent*	*Number*	*Percent*	*Number*	*Percent*	*Number*	*Percent*	*Number*	*Percent*	*Number*	*Percent*
12	45	100	10	90	8	88	15	100	55	0	60	0
13	45	100	10	100	8	100	15	100	73	0	75	0
14	45	98	10	100	8	100	15	100	75	0	75	0
15	45	100	10	100	8	100	15	100	74	0	60	0
16	45	42	10	80	8	25	15	100	75	0	75	0
17	45	100	10	100	8	88	15	100	75	0	75	0
18	45	98	10	100	8	88	15	100	57	0	60	0
19	45	100	10	100	8	88	15	100	70	0	75	0

Single hyphal-tip lines were secured from isolates 12 to 19. Three lines were secured from isolate 15; two lines each from isolates 13, 16, 17, and 19; and one line each from isolates 12, 14, and 18. They were increased on cornmeal-sand medium and introduced into clean soil. Two homozygous susceptible lines and one homozygous resistant line of cabbage were tested. The results, given in table 4, again show no evidence of infection of the resistant line. The data in table 3 show reduced virulence in the original isolate 16 but not in isolate 15. It is apparent, however, that all the lines derived from these isolates 15 and 16 showed reduced virulence upon the susceptible strains of cabbage. This is the only evidence obtained in this study of any marked variation in virulence. It is to be noted, however, that it was variation in the direction of lower virulence on susceptible cabbage and not in the direction of increased virulence upon resistant cabbage.

TABLE 4.—*Pathogenicity of hyphal-tip lines from isolates of Fusarium conglutinans upon homozygous susceptible and homozygous resistant strains of cabbage*

Hyphal-tip line	Plants of homozygous susceptible strain—				Plants of homozygous resistant strain 40-28-A			
	C-29-A		SP-GC		Trial 1		Trial 2	
	Total	Diseased	Total	Diseased	Total	Diseased	Total	Diseased
	Number	*Percent*	*Number*	*Percent*	*Number*	*Percent*	*Number*	*Percent*
12-1	12	100	13	100	12	0	10	0
13-1	12	92	13	100	12	0	10	0
13-2	12	92					10	0
14-1	12	100	13	100	12	0	10	0
15-1	12	83	13	46	12	0	10	0
15-2	12	58					10	0
15-3	12	50					10	0
16-1	12	8	13	23	12	0	10	0
16-2	12	25					10	0
17-1	12	100	13	100	12	0	10	0
17-2	12	100					10	0
18-1	12	92	13	92	12	0	10	0
19-1	12	100	13	100	12	0	10	0
19-2	12	100					10	0

TRIALS WITH F_2 HYBRIDS FROM CROSSES BETWEEN RESISTANT AND SUSCEPTIBLE LINES OF CABBAGE

The inheritance of resistance in cabbage to the yellows organism, *Fusarium conglutinans*, has been found to be based upon a single dominant factor (*4*). F_2 hybrids of crosses between resistant and susceptible plants segregate in the second generation in the ratio of 3 resistant to 1 susceptible. The availability of seed led to a study of the reaction of 4 F_2 progenies to 9 of the most virulent isolates of the fungus. It is to be seen from the results in table 5 that all progenies segregated very closely in the expected ratio of 3 resistant to 1 susceptible when exposed to each isolation of the yellows organism.

TABLE 5.—*Pathogenicity of 9 isolates of Fusarium conglutinans upon F_2 hybrids from resistant susceptible crosses*

Isolate no.	F_2 strain											
	5HIA			H5ID			C20I-1s			G5IC		
	Healthy plants	Diseased plants	Deviation,* probable error	Healthy plants	Diseased plants	Deviation, probable error	Healthy plants	Diseased plants	Deviation, probable error	Healthy plants	Diseased plants	Deviation, probable error
	Number	Number		Number	Number		Number	Number		Number	Number	
1	46	9	2.2	43	15	0.2	38	14	0.5	12	4	0
2	42	16	.7	48	14	.7	38	14	.5	13	3	.9
4	42	15	.3	47	10	1.9	39	13	0	20	10	1.6
5	46	15	.1	49	11	1.8	40	12	.5	21	9	.9
6	40	19	1.9	40	19	1.9	40	12	.5	23	7	.3
8	46	15	.1	45	15	0	41	11	.9	22	8	.3
9	46	12	1.1	44	16	.4	37	15	.9	12	4	0
10	46	15	.1	41	19	1.8	39	13	0	23	7	.3
11	44	17	.8	43	16	.6	39	13	0	25	5	1.6

* Deviation from the expected segregation of a monohybrid, i.e., 3 healthy to 1 diseased.

TRIALS WITH CERTAIN SUBSPECIES OF BRASSICA OLERACEA

Eight of the isolates were tested upon New Snowball cauliflower, White Vienna kohlrabi, Moss-Curled and Thousand-Headed kale, and Copenhagen Market cabbage. All these were commercial lots of seed and were therefore not expected to be uniform for resistance or susceptibility. Walker and Wellman (*7*) in field trials showed these subspecies to contain varying percentages of resistant individuals when commercial lots were used. The results of this trial (table 6) show that each subspecies contained a high percentage of individuals susceptible to each isolate. There are some fairly wide variations in the percentage of individuals of a given variety infected by the various isolates. This may be accounted for by the fact that the population of a given variety was homozygous neither for resistance nor susceptibility, and therefore a much larger number of test plants in each case would be necessary to establish the significance of such differences. It is clear, however, that all varieties of the subspecies used were relatively highly susceptible to the isolates used. Similar trials with Long Island Improved brussels sprouts and Georgia collards gave corresponding results. In none of these tests was there any evidence

of selective pathogenicity for one or more of the subspecies of *Brassica oleracea*.

TABLE 6.—*Pathogenicity of 8 isolates of Fusarium conglutinans upon subspecies of Brassica oleracea* [a]

Isolate no.	Diseased plants in—				
	Cabbage	Cauliflower	Kohlrabi	Moss-Curled kale	Thousand-Headed kale
	Percent	*Percent*	*Percent*	*Percent*	*Percent*
1	90	67	57	85	62
4	92	85	64	92	58
5	100	100	71	100	80
6	100	77	69	92	52
8	85	75	67	67	85
9	85	75	67	77	50
10	100	100	71	94	59
11	92	58	53	92	61

[a] The number of plants in each test of each isolate ranged from 10 to 23.

CULTURAL STUDIES

Throughout the routine transfer of the cultures of the fungus and the frequent reisolations from diseased plants it was noted that the isolates resembled one another closely in gross cultural characteristics. Certain experiments were conducted to compare more accurately their cultural characters. The temperature range over which the cultures were observed was from 20° to 28° C. The isolates were compared as to rate of colony growth, spore production, and pigment formation. The media used were malt-extract agar, potato-dextrose agar, steamed rice, oatmeal agar, and Richards' nutrient solution.

The rate of growth of the fungus, as measured by increase in colony diameter, was studied on potato-dextrose and malt agars in Petri dishes. Precautions were taken to insure equal conditions for the comparative studies, and the plates of a single isolate were made in triplicate. Isolates 1 to 11 were studied as one group; isolates 12 to 19 and the hyphal-tip lines derived from them were compared later. The growth rates of all fell within fairly narrow limits. The optimum temperature for all appeared to be that commonly reported for *Fusarium conglutinans*, namely, about 25° to 27° C. All displayed similar gross characteristics as to character and amount of aerial mycelium. It was noted, however, that the colony margin of isolate 10 was more sharply defined than that of the others, and that the aerial growth of isolate 16 was somewhat less abundant than the aerial growth of the others. Although the cultural characters of isolate 15 were typical of those commonly described for *F. conglutinans*, the hyphal-tip lines derived from it displayed considerable variation in the character and amount of aerial growth. The cultures were compared as to color production upon the above-mentioned agars and upon oatmeal agar, steamed rice, and Richards' nutrient solution. None produced color upon any of the media except steamed rice, where a salmon-pink color was produced by all. Microconidia and chlamydospores were produced by all, whereas macroconidia were found only occasionally. Saltations were not uncommon in the hyphal-tip lines of isolate 15, whereas the other collections were apparently very stable and failed to show this type of variation.

DISCUSSION

Cultural studies of a number of isolations of the cabbage-yellows organism failed to bring out significant differences in their behavior. These studies included comparisons as to rate of growth, color production, and sporulation. It is not the writer's contention that the isolates of the fungus that were studied are identical, since certain minor differences in their behavior were evident. The variations that appeared during the cultural work are being studied further to determine whether or not they are of significance. During the routine handling and observation of the cultures there has been evidence of sectoring only in the hyphal-tip lines of one of the isolates. In general the numerous isolates of the organism have been remarkably uniform in their behavior.

The data accumulated in the pathogenicity trials upon selected lines of cabbage and upon other subspecies of *Brassica oleracea* are in accord with the general results of the cultural studies. The isolates gave one general type of reaction in which all concur. Lines of cabbage selected for susceptibility were uniformly attacked by all except isolate 16 and the hyphal-tip lines from isolate 15, which differ only in showing a reduced degree of virulence toward susceptible plants.

Further evidence of the uniformity of the isolates is presented in the comparative pathogenicity trials upon the F_2 hybrids, in all of which a close fit to the expected 25 percent of diseased plants was obtained. No selective pathogenicity was displayed in trials with other subspecies of *B. oleracea*. Selected lines of cabbage that were homozygous for resistance showed no symptoms of the yellows disease when exposed to each isolate at a soil temperature of 24° C.

Although strains of *Fusarium conglutinans* may occur which do not conform in their pathogenic properties to those studied, the wide geographic range covered by this collection of cultures strongly indicates that this species is very stable in its selective pathogenicity. There is reason to believe, therefore, that the varieties of the various subspecies of *Brassica oleracea* selected for resistance in one locality will remain stable for this character in other localities.

SUMMARY

A study was made of the comparative pathogenicity and cultural behavior of 19 isolates of the causal organism of cabbage yellows, *Fusarium conglutinans* Wr., from 11 States.

Comparative cultural studies of the 19 isolates and hyphal-tip lines derived from certain of them were made as to growth rate, spore production, and color production. The growth rates of all were similar when tested over a series of temperatures, no outstanding differences being noted. Microconidia and chlamydospores were produced by all isolates; macroconidia were not consistently present. On steamed rice all isolates produced a salmon-pink to flesh color after a week or more in strong diffused light. Sectoring was present only in the hyphal-tip lines of one isolate.

Homozygous susceptible lines of cabbage were uniformly attacked by all except two of the isolates, in which the organisms were less virulent upon homozygous susceptible cabbage.

All isolates of the fungus were alike in their inability to attack successfully the homozygous resistant lines of cabbage at 24° C.

In the F_2 hybrid lines of resistant-susceptible crosses the percentage of plants that became diseased when tested against nine isolates was in all cases close to the expected 25 percent.

The isolates showed no evidence of selective pathogenicity upon the six subspecies of *Brassica oleracea* that were studied.

From the results presented in the foregoing data it is safe to assume that specialization is not a vital factor in the program of selection and breeding for resistance to the cabbage-yellows pathogene.

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TOXIN PRODUCED BY BACTERIUM TABACUM AND ITS RELATION TO HOST RANGE[1]

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INTRODUCTION

Since 1918, when the wildfire disease of tobacco (*Nicotiana tabacum* L.) was originally described by Wolf and Foster (*13*),[3] reports of its host range have varied from tobacco only to a large number of species in many genera. Wolf (*12*) and Clinton and McCormick (*3*) considered the causal organism, *Bacterium tabacum*,[4] as limited to tobacco species. Chapman and Anderson (*2*), however, reported infection on eggplant, pokeweed, and petunia, and observed naturally occurring lesions on tomato. In 1924 Johnson, Slagg, and Murwin (*8, p. 180*) reported results from inoculation experiments in which they secured infection on species of 23 genera with indications that "the host range of *Bact. tabacum* is very much larger than indicated by these results." In 1929 Săvulescu and Radulescu (*11*) reported a study of a serious tobacco disease in Rumania which they ascribed to *Bact. melleum* Johnson, but which appears from their publication to be much more like wildfire. A feature of their report was a very extensive host range, with conclusions as to susceptibility of various families and species, which correspond closely with those reported by Johnson, Slagg, and Murwin (*8*).

A complicating factor, however, was introduced by the discovery of Johnson and Murwin (*7*) in 1925 that *Bacterium tabacum* secretes a toxic substance in culture, and that this substance when filtered free from bacteria and inoculated into leaves produces typical symptoms of the wildfire disease. Johnson and Murwin point out that this might raise a question as to the previously described host range, but conclude that "in most cases at least the organism was actively parasitic, although the initial symptoms may have been produced by toxin introduced from cultures."

The present study was undertaken as part of a general investigation of the tobacco leaf-spot diseases.

TOXIN PRODUCED BY BACTERIUM TABACUM IN PURE CULTURE

Bacterium tabacum grows well on a variety of media, both liquid and solid, and produces toxin freely on all media tested. The usual potato-dextrose broth proved well adapted for the experimental work, and was used throughout the tests. As previously indicated, the toxin can be secured free from bacteria by filtration, and the same

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[3] Reference is made by number (italic) to Literature Cited, p. 425.

[4] Synonym: *Phytomonas tabaca* (Wolf and Foster) Bergey et al.

result can be secured by heat or chemical sterilization. It has been possible to demonstrate by these different methods that as early as 24 hours after transfer toxin in appreciable amounts is present in culture tubes incubated at 20° to 25° C. Cultures 48 hours old contain sufficient toxin to produce large leaf lesions, and cultures 5 to 7 days old may be diluted with water up to 16 times and still give a strong positive reaction when inoculated into the leaf. The accumulation of toxin in liquid culture gradually ceases, and tests with cultures 6 months old have shown no toxin, although living bacteria were still present. That the bacteria in these cultures had actually destroyed their own toxin was proved by the fact that similar cultures sterilized when 2 weeks old contained an abundance of toxin at the end of the 6 months' storage.

PROPERTIES OF TOXIN

The fact that cultures can be freed from the bacteria by passage through a Berkefeld filter and that the toxin is present in the filtrate has been established by Johnson and Murwin (7). This work was repeated and the results confirmed. In addition, collodion, collodion plus 4 percent glycerin, and cellulose sacks were prepared. These three types of sacks were tested by filling with water, and all showing any signs of defect were rejected. The selected sacks were then filled with (1) a sterile Berkefeld filtrate and (2) 3-day-old *Bacterium tabacum* cultures. The filtrate secured from the sacks was inoculated into leaves, and in each case the presence of abundant toxin was demonstrated. Agar dilution plates were poured from the culture filtrate (2) and also from inoculated plants and no *Bact. tabacum* bacteria were secured. These results indicated that the toxin was actually excreted by the bacteria, that it could be secured without destroying the bacteria, and that the toxin particles were very small.

Since the toxins known to animal pathology and also the virus causing mosaic disease of tobacco can be precipitated readily by lead acetate, the action of this substance and that of calcium acetate on the *Bacterium tabacum* toxin was tested. The procedure followed with neutral lead and calcium acetates was to add an excess of a 5-percent solution to separate portions of the filtrate from a 12-day-old broth culture. This treatment raised the pH value from an original 4.3 to about 5. As there was no precipitate in the portion treated with calcium acetate, this portion was held without further treatment. The portion treated with lead acetate showed a thick gellike precipitate which was removed by centrifuging, and the clear liquid retained. The excess of lead was precipitated with sulphuric acid and removed, and the clear liquid was brought to a pH of 6.6 with potassium hydroxide. Leaf-prick inoculations were made with (1) the original culture-solution filtrate, (2) the portion treated with calcium acetate, (3) the clear liquid left after treating a portion of one with lead acetate, and (4) the same after precipitating the excess of lead and restoring to a pH of 6.6. The inoculation results were promptly and strongly positive with each lot, indicating that none of the treatments had affected the toxin.

Diluting a culture solution with an equal volume of absolute alcohol did not precipitate or destroy the toxin. The alcohol mixture gave positive results on inoculation, as did also the clear solution remaining

after the alcohol had been driven off by heat and the precipitate removed. After a culture solution had been boiled with bone black and filtered, the toxin was still present in the filtrate.

Sulphuric and hydrochloric acids and sodium and potassium hydroxides were used to test the action of acids and alkalies. The toxin was completely and rapidly inactivated by 0.25 percent of sodium or potassium hydroxide, and furthermore the inoculated leaves developed no infections even after a month or more, indicating destruction of both toxin and bacteria. Ammonium hydroxide of similar strength, however, did not affect the toxin. Potassium carbonate, a basic salt, destroyed the toxin, though less rapidly than did the hydroxide, and a stronger solution was required. The action of alkalies on the toxin was not instantaneous, but took place over a considerable period as indicated in table 1. The action of the alkalies was not reversible, since solutions of toxin inactivated by treatment with sodium hydroxide and then restored to their original pH by adding hydrochloric acid continued to give negative results. Neither hydrochloric nor sulphuric acid of strength up to 0.5 percent inactivated the toxin.

TABLE 1.—*Results from inoculations with toxin solutions to which various chemicals had been added*

[15 inoculations were made for each test]

Chemical added to toxin solution	Strength of chemical	Inoculations made—			
		1 hour after treatment		24 hours after treatment	
		Negative	Positive	Negative	Positive
	Percent	*Number*	*Number*	*Number*	*Number*
KOH	0.10	11	4	15	0
	.15	12	3	15	0
	.20	15	0	15	0
	.25	15	0	15	0
	.30	15	0	15	0
NaOH	.25	15	0	15	0
K_2CO_3	.25	0	15	0	*15
	.50	9	6	15	0
	1.00	9	6	15	0
Untreated		0	15	0	15

* Very faint.

It is to be noted that the weaker strengths of the hydroxide (0.1 and 0.15 percent) were completely effective in destroying the toxin after 24 hours and only partly effective after 1 hour. The value of the longer treatment was also evident with the potassium carbonate, which in strengths of 0.5 and 1 percent was completely effective after 24 hours and only partly effective after 1 hour. These and similar results would seem to indicate a slow breaking down of the toxin by the alkali rather than an instantaneous reaction.

To test the effect of a powerful disinfectant, 10-day-old cultures were treated so as to give 0.0375, 0.075, 0.15, and 0.3 percent solutions of mercuric chloride. After 12 hours tobacco leaves were inoculated with these solutions. Transfers were made from each treated lot to tubes of sterile broth, to check for the presence of living bacteria. The leaf inoculations gave strong positive results, indicating an abun-

dance of toxin in all. The transfers showed that even the lowest mercuric chloride concentration had destroyed all bacteria. Similar tests were conducted with formaldehyde, and here again the bacteria were readily destroyed by comparatively low concentrations, but the toxin was not affected.

It was early found that this toxin had very high thermal stability. Originally temperatures of 50° to 60° C. were used, with exposures of 10 to 60 minutes, the plan being to find an exposure that would destroy the bacteria but not injure the toxin. On investigation, however, it was discovered that even steaming in the autoclave for 30 minutes with 15 pounds pressure did not inactivate the toxin, nor did boiling over a free flame for 3 hours, during the course of which the volume of the solution was reduced 87 percent. It was shown, however, by testing the rediluted solution, that this prolonged boiling had destroyed a part of the toxin.

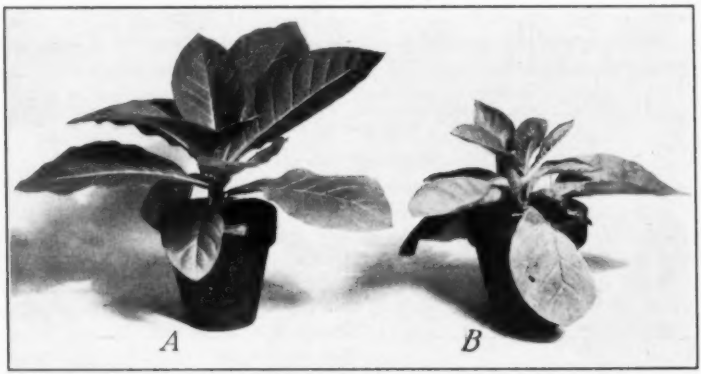

FIGURE 1.—Tobacco plants, showing effect of toxin of *Bacterium tabacum* on entire plant after inoculation of vascular tissues of stem: *A*, Uninoculated plant used as check; *B*, plant 6 weeks after inoculation of stem; the entire plant was yellowed and stunted, although the bacteria were present only in a small area around the point of inoculation.

ACTION OF TOXIN ON PLANT TISSUES

The wildfire leaf lesion is very characteristic in appearance, so much so that the organism is usually identified by inoculating leaves and watching for the development of the large yellow halo spots. Their circular shape indicates a diffusion of some substance out from the central point of infection, and investigation showed that the color of the halo was due to destruction of the green chlorophyll, which permitted the yellow carotene pigment to become visible. The chlorophyll of many plants was so sensitive to this toxin that a strong solution applied to uninjured leaves caused the treated areas to turn a yellow green. Additional proof of the very powerful action of the toxin on chlorophyll was supplied by inoculating greenhouse tobacco plants in the stem (fig. 1). The leaves above the inoculation turned yellow, though the actual infection usually remained confined to a small area near the point of inoculation. Indications of a definite chemical reaction between the toxin and chlorophyll were supplied by experiments in which a fresh alcohol extraction of chlorophyll and a culture of *Bacterium tabacum* were mixed half and half. After

several days a distinct precipitate was formed which on settling out left a yellow-green liquid in place of the original green. Similar tests with an organism such as *Bact. angulatum* Fromme and Murray, which secretes no such toxin, gave no reaction.

Further evidence of a specific action of the toxin on chlorophyll was supplied by the following experiment: Lots of tobacco and bean-leaf tissue were extracted with alcohol overnight, and then liquid cultures of *Bacterium tabacum* were treated with equal portions of (1) pure alcohol and (2) the alcoholic chlorophyll extracts. After standing 5 days, the alcohol was evaporated by heating and leaves were prick inoculated. The halos obtained with 1, the alcohol-treated portion, were very large and clear; but with 2, the alcohol-chlorophyll-

FIGURE 2.—String bean plants showing extreme susceptibility to the toxin of *Bacterium tabacum*. *A*, Check plants sprayed with water. *B*, Plants sprayed with ordinary liquid culture of *Bact. tabacum* diluted with an equal volume of water; plants then held at 55° to 65° F. *C*, Plants treated as in *B* but held at 70° to 80°. Similar results were secured by means of sterile cultures. Tobacco plants treated in same manner showed a slight yellowing of some leaves but suffered no permanent injury from the toxin.

treated lots, only negative to faintly positive results were secured, indicating that the toxin had been largely inactivated.

The chlorophyll of many kinds of plants is attacked by this toxin, and wide variations in susceptibility exist. Tobacco is only moderately susceptible, whereas the string bean (*Phaseolus vulgaris* L.) is extremely susceptible. Thus bean plants may be completely killed by spraying with a toxin solution that only slightly injures tobacco plants (fig. 2). The action of the toxin is subject to the usual temperature laws, being greatest at high temperatures.

GREENHOUSE EXPERIMENTS

INOCULATIONS

Owing to the difficulty of properly controlling conditions in the field, most inoculation experiments are conducted in the greenhouse, and this has been true of the previous work with *Bacterium tabacum*.

The writer's experiments have covered a period of 2 years, and large numbers of tests under a wide variety of conditions have been made. The results secured are summarized, since a presentation of the separate data would require far too much space. The following is a brief statement of the methods employed.

Wound inoculations were made with glass tubes drawn to a point and broken off to give a small opening. The tube was filled with the inoculating liquid, and, as a prick was made, a drop of the fluid was left on the leaf. Spray inoculations were made with the usual atomizers, and in either case the plants were placed in a damp chamber for 3 to 5 days after inoculation. The work was generally conducted at temperatures between 70° and 80° F. Since the usual culture contained both bacteria and toxin, it was thought necessary, in addition to inoculating with such cultures, to make comparative tests with the bacteria and toxin separately. Bacteria free from toxin were secured by centrifuging cultures, pouring off the supernatant liquid, resuspending the precipitate in distilled water, and repeating this procedure twice. The suspension of bacteria so obtained gave no test for toxin if heated and inoculated, and showed an abundance of living bacteria as evidenced by microscopic examination and by heavy infection resulting from spray or wound inoculations. Owing to its stability, it was very easy to secure solutions of the toxin free from bacteria by sterilizing with heat and filtering through a Berkefeld, or by treating with 0.05 percent mercuric chloride.

It was soon apparent that short incubation periods of 2 to 3 days obtained with wound inoculations were largely the result of toxin contained in the inoculating fluid. This quick appearance of infection was secured either with the culture solution containing both toxin and bacteria or with the toxin alone. When bacteria alone were used on tobacco leaves in a susceptible condition, usually about 7 days were required before positive results were apparent (fig. 3). The subsequent development of the disease, however, was the same, and after 3 to 4 weeks the lesions secured with bacteria or with bacteria plus toxin were similar. The lesions from the toxin alone, on the other hand, soon ceased to develop, and after a few weeks were less conspicuous than the others, indicating that with tobacco the first symptoms produced by the toxin of the inoculating fluid are supplemented by further symptoms as the result of toxin produced by the bacteria in the leaf tissues.

Inoculations were made on many other plants, including cucumber (*Cucumis sativus* L.), cantaloup (*Cucumis melo* L.), bean (*Phaseolus vulgaris* L.), *Begonia* sp., eggplant (*Solanum melongena* L.), bindweed (*Convolvulvus arvensis* L.), *Geranium* sp., lantana (*Lantana camara* L.), oat (*Avena sativa* L.), petunia (*Petunia hybrida* Hort.), pepper (*Capsicum annuum* L.), poinsettia (*Euphorbia pulcherrima* Willd.), tomato (*Lycopersicon esculentum* Mill.), mustard (*Brassica nigra* (L.) Koch), and zinnia (*Zinnia elegans* Jacq.). The results from inoculations with toxin or with toxin plus bacteria were positive. In all of these the lesions appeared promptly and were large and characteristic of the wildfire disease (figs. 4 and 5). In the case of some of these plants the lesions appeared very quickly and were in every way larger and more conspicuous than those secured in the comparative inoculations on tobacco. Positive results of this sort, however, were never secured from inoculations with the bacteria alone. Not infre-

quently a narrow margin of dead tissue developed around the inoculation wound and, in an extremely toxin-susceptible plant like the bean, small halo lesions occasionally appeared. There was, however, no subsequent development of the lesions, and it is a significant fact that with all plants other than tobacco the lesions, whatever the inoculum used, developed to a maximum size within 10 days, whereas with tobacco they often continued to enlarge over a period of weeks. This difference in behavior is to be associated with information presented later on the ability of the organism to persist and multiply in the tissues of various plants. The main result from the extensive series of wound-inoculation experiments was the fact that whereas tests with toxin or toxin plus bacteria indicated that there were many plants susceptible to the disease and some of them very much more

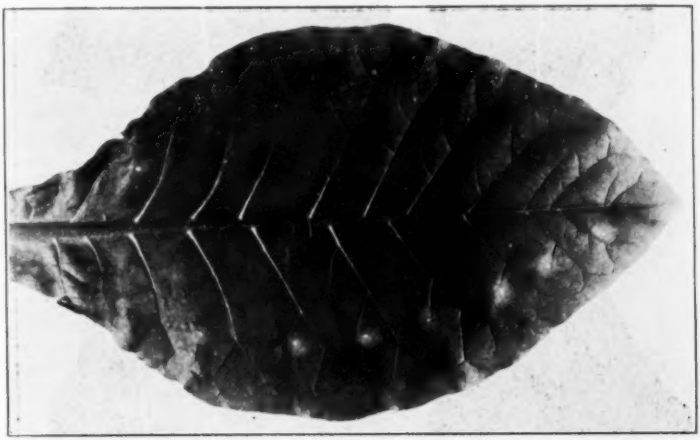

FIGURE 3.—Tobacco leaf inoculated with *Bacterium tabacum*. The lower half was wound-inoculated with an 8-day-old culture containing an abundance of bacteria and toxin; the upper half, with bacteria washed free from toxin. Photograph taken 8 days after inoculation, when halo lesions resulting from bacteria plus toxin ranged in diameter up to three eighths of an inch, with a fringe of dead tissue immediately around the central prick. Inoculations with bacteria alone were just beginning to show a faint yellowing. Three weeks later lesions resulting from bacteria plus toxin and those from bacteria alone were equally well developed.

susceptible than tobacco, as judged by the size of lesions secured, tests with the bacteria alone failed to give positive results, except with tobacco.

In addition to the wound inoculations, many tests were made by atomizing the leaves with diluted cultures, or suspensions of bacteria free from toxin. Other investigators have noted that this is a less certain method of obtaining infection than wounding. However, it was found that by providing suitable conditions good infection could be secured on uninjured tobacco leaves. Inoculations on other plants generally gave no infection, but by repeated and extensive tests it was found that occasionally some lesions could be secured. Such lesions were obtained on cantaloup, cucumber, eggplant, and tomato. The difference in the results from spray inoculations on these plants as compared with those on tobacco was in the amount of infection. Thus with conditions so favorable as to give up to 500 infections on

individual tobacco leaves, 2 to 5 infections might result on the inoculated leaves of some of the other plants.

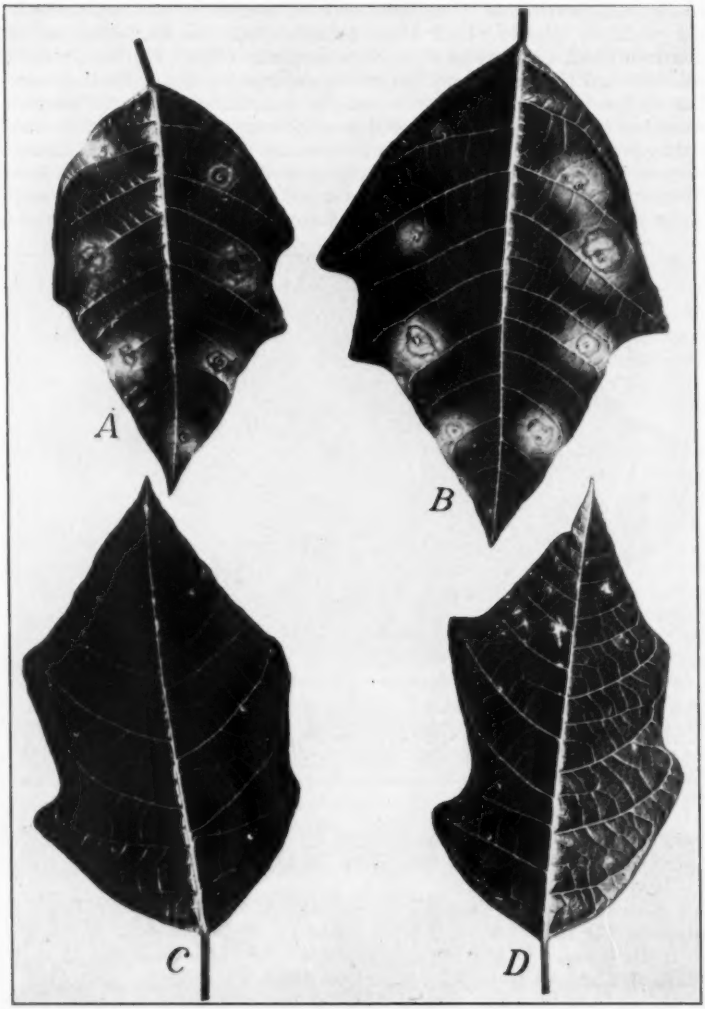

FIGURE 4.—Poinsettia leaves inoculated at same time and in same manner as tobacco leaf (fig. 3). *A* and *B*, Leaves inoculated with bacteria plus toxin; lesions ranged in diameter up to five eighths of an inch; central dead areas ranged in diameter up to three eighths of an inch. *C* and *D*, Leaves inoculated with bacteria free from toxin; some pricks had a narrow fringe of dead tissue, but there was no further development of lesions, even after 6 weeks. Note that the striking results on *A* and *B*, without the check on *C* and *D*, would indicate that the poinsettia is more susceptible than tobacco to *Bacterium tabacum*.

ISOLATIONS

In previous work on host range of *Bacterium tabacum* investigators in some instances have regarded the appearance of the characteristic

leaf lesions as positive proof of infection; in other cases they have reisolated the organism and proved its pathogenicity by reinoculation, thus completing the proof as prescribed by Koch. The present writer has found that after wound inoculations it is quite possible to reisolate the organism from many plants, but that the longer this reisolation is delayed the more difficult it becomes, although leaf symptoms may continue prominent. The writer's tests indicated that in plants other than tobacco and susceptible species of *Nicotiana* the bacteria effect a temporary lodgment, but instead of multiplying and spreading they rapidly decline in number. The difference in the number of bacteria present 3 weeks after inoculation in tobacco, as compared with that in other plants, was readily shown by poured plates. The procedure followed was to remove with a small cork borer equal areas from lesions

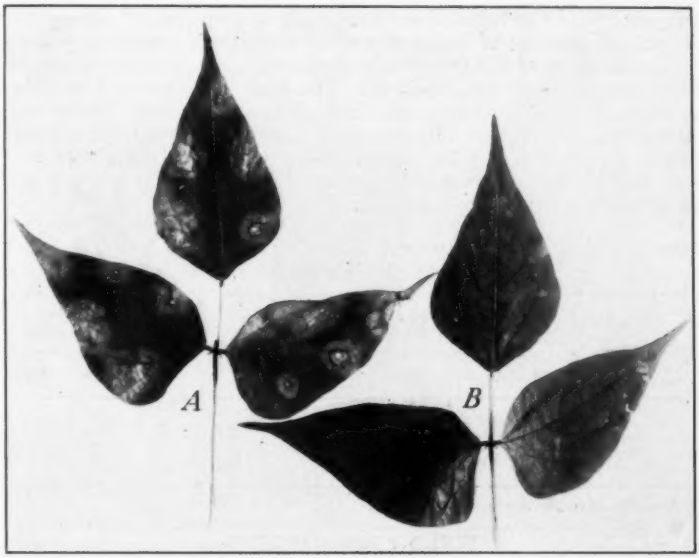

FIGURE 5.—Bean leaves inoculated at same time as tobacco leaf (fig. 3). *A*, Leaves inoculated with toxin; lesions ranged in diameter up to 1¼ inches; central dead areas ranged in diameter up to one half inch. *B*, Leaves inoculated with bacteria; few small lesions, which made no further development. Bean-leaf inoculations with toxin alone frequently gave even larger lesions than did inoculations with toxin plus bacteria.

of similar age, wash in sterile water, crush, and pour dilution plates. An example of the data so secured is given in table 2.

TABLE 2.—*Dilution-plate counts from tissues of plants inoculated with Bacterium tabacum*

Dilution plate no.	Colonies of *Bact. tabacum* from tissues of—			
	Tobacco	Tomato	Cucumber	Bean
	Number	*Number*	*Number*	*Number*
1	418	39	93	35
2	379	24	91	36
3	78	2	49	11
4	19	1	3	4

The data in table 2 were obtained during a period of medium temperatures (65° to 75° F.). Plants were also inoculated and held at temperatures of 55° to 65°, with results indicating that the bacterium in plants other than tobacco can survive longer under these cooler conditions. On the other hand, during very warm weather, even after 3 weeks, it was often impossible to reisolate the organism from any plant except tobacco. It has been previously stated that some plants are much more sensitive to the action of the toxin than tobacco, and the bean is especially so. The sensitivity of the plant to the toxin, however, and the ability of the organism to establish itself and to multiply in the plant tissues seem to have no relation to each other. Thus from inoculations made at the same time on bean and tobacco the lesions on the bean leaves were much larger than those on tobacco, so that on the basis of symptoms one would classify the bean as highly susceptible and the tobacco as moderately susceptible. Consequently the general practice of measuring susceptibility of plants to a disease by the intensity of the symptoms produced appears to be misleading in the case of *Bacterium tabacum*. The bean, which is susceptible to the toxin, is a most unfavorable food plant for the bacteria (table 3), and hence the ability of this organism to establish parasitic relationships is dependent not on susceptibility of the plant tissues to its toxin, but rather on some unknown quality which favors the growth and multiplication of the bacteria.

TABLE 3.—*Dilution-plate counts from tissues of tobacco and bean 4 weeks after inoculation with Bacterium tabacum*

Dilution plate no.	Colonies of *Bact. tabacum* from tissues of—		Dilution plate no.	Colonies of *Bact. tabacum* from tissues of—	
	Tobacco	Bean		Tobacco	Bean
	Number	*Number*		*Number*	*Number*
1	(*)	16	4	450	0
2	(*)	5	5	63	0
3	1,000+	1	6	30	0

* Colonies too numerous to count.

FIELD EXPERIMENTS

INOCULATIONS

In order to study the occurrence of wildfire disease under field conditions, plantings of tobacco were made with a number of other crops intermingled. Some of the plants used in this manner were cantaloup, cucumber, bean, eggplant, pepper, tomato, sweetpotato (*Ipomoea batatas* (L.) Lam.), potato (*Solanum tuberosum* L.), watermelon (*Citrullus vulgaris* Schrad.), chrysanthemum (*Chrysanthemum coronarium* L.), snapdragon (*Antirrhinum majus* L.), sweet alyssum (*Alyssum maritimum* L.), coleus (*Coleus blumei* Benth.), and zinnia. To inoculate the plantings uniformly the bacteria were grown in liter flasks and the diluted cultures applied with an ordinary compressed-air sprayer. Inoculations were made during rains at intervals through the season, and infection consisting of a few scattering lesions was secured at various times on cucumber, cantaloup, bean, pepper, and zinnia (fig. 6). The difference between the response of

these plants and that of tobacco, however, was twofold: (1) The number of lesions secured on tobacco was very much larger than on any other plant; (2) with tobacco there was a continuous spread of the disease from infected to healthy leaves; with other plants there were occasional lesions without subsequent spread.

ISOLATIONS

Isolations and dilution plates from the lesions secured in the field plots gave results comparable to those already described for the greenhouse inoculations, except that whereas the plates from tobacco spots usually gave practically a pure growth of *Bacterium tabacum*,

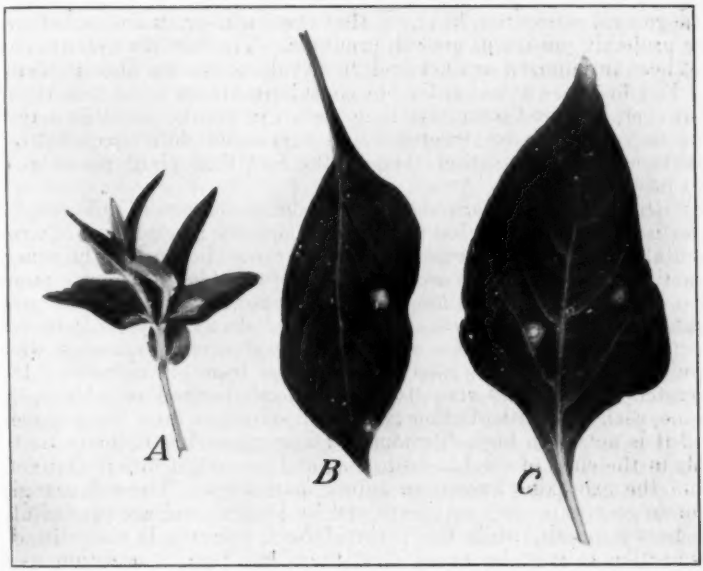

FIGURE 6.—Zinnia (*A*) and pepper (*B* and *C*) leaf lesions resulting from field spray inoculations with *Bacterium tabacum*. Such occasional infections were secured with a variety of plants, and in some instances, at least, were not associated with wounds of any sort.

similar plates from the lesions on other plants often gave a very mixed bacterial growth.

SUSCEPTIBILITY OF SPECIES OF NICOTIANA

During the course of these experiments many varieties of *Nicotiana tabacum* and certain other *Nicotiana* species were successfully inoculated with *Bacterium tabacum*. No attempt was made at this time to resurvey the field covered by Anderson (*1*) in his study of the susceptibility of *Nicotiana* species. The methods adopted in his inoculations obviated completely the possibility of misleading toxin effects.

DISCUSSION

The subject of toxins has received attention from plant pathologists in recent years, and the existence of toxic materials has been demonstrated in studies of certain wilt diseases by Young and Bennett (*14*), Fahmy (*4*), Goss (*5*), Rosen (*10*), and others. These results indicated that the fungi concerned produced poisonous substances which were the direct cause of the wilting, this view replacing the earlier conception of a mechanical plugging of the water-conducting tissue. These materials were mostly thermostable. In certain cases it was shown that the wilt-producing toxin of a particular parasite was effective in wilting other plants than the usual host. Likewise a given plant may be wilted by products from other fungi than the usual parasite. The general conception has been that these wilt-producing substances are probably poisonous growth products. The fact that plants have not been immunized against such toxic substances has also been cited by Picado (*9*) as a reason for not considering them to be true toxins. However, acquired immunity is unknown in plants, and this may be due to certain basic structural and functional differences between plants and animals rather than to the fact that plant parasites do not produce toxins.

With *Bacterium tabacum* and the wildfire disease of tobacco, the question of toxic action has assumed importance in studies of environmental relations, overwintering, and host range, because of the general practice of inoculation by wounding and of regarding the development of a characteristic lesion as proof of infection. Studies of the properties of the *Bact. tabacum* toxin indicated that it is present in very young cultures and hence is not a gradual accumulation of waste products. In fact, it is likely to disappear from old cultures. It is excreted by the bacteria. It has a specific action on chlorophyll tissue, with the death of other tissues following as a later development, and it is active in high dilutions. These properties indicate that it falls in the class of soluble exotoxins, and as such it differs distinctly from the exotoxins known to animal pathology. These latter give protein reactions, they are destroyed by heating and are precipitated by heavy metals, while the toxin of *Bact. tabacum* is not. In this connection it may be noted that there has been a question as to whether the animal-parasite exotoxins are actually proteinlike or are merely closely linked to protein, and hence whether a pure exotoxin would actually react like protein.

Proof of the pathogenicity of an organism is generally based on the well-known Koch postulates. Pathologists conducting studies of host range, however, have frequently regarded the production of typical disease symptoms after pure-culture inoculation as proof of a parasitic relationship, and this procedure was followed in some of the previous work with *Bacterium tabacum*. This practice rests on the assumption that the lesions must be the result of parasitic activity of the bacteria in the plant tissues. The writer's experiments show, however, that with the usual method of wound inoculation it was possible to produce characteristic wildfire lesions on the leaves of most of the plants tested, and that these results could be secured with solutions containing the bacterial toxin whether or not the bacteria were present. Similar tests with bacteria washed free from toxin, however, consistently produced typical wildfire lesions only on

tobacco. Further investigations indicated that the degree of susceptibility to the toxin was a stable property varying widely with different species of plants. With the bean, a highly toxin-susceptible plant, the lesions ranged up to 1 inch in diameter and the dead central area was surrounded by a broad halo band. Lettuce (*Lactuca sativa* L.) was nearly nonsusceptible, and the inoculated leaves showed only faint halo spots. Tobacco proved to be only very moderately toxin-susceptible. The following explanation of the mechanics of these lesions is suggested.

The wound inoculations provided entrance into the leaf for a small amount of the toxic substance, which diffused out from the central point into the tissues. The leaf chloroplasts appeared to be most sensitive to the toxin action, and were destroyed first. The disintegration of the chlorophyll made the yellow carotene pigment visible, and hence a yellow halo spot was formed. With many plants this was the extent of the lesion, but with certain very susceptible plants the tissues in the center that received the most toxin were killed entirely, and the yellow halo then appeared around this central dead area. These lesions, therefore, were fundamentally chemical in nature and not a proof of parasitism, since various chemicals similarly pricked into leaves would result in producing lesions of various types, and differences in the susceptibility of species could doubtless be established. Final proof that susceptibility to the *Bacterium tabacum* toxin and susceptibility to parasitic attack by the bacteria are not correlated is supplied by the fact that the wound inoculations with toxin and bacteria used separately give very different results, the conclusion being that many plants are susceptible to the action of the toxin while only tobacco is susceptible to attack by the bacteria.

Infection as it occurs under natural conditions, of course, is not complicated by toxin effects, such as occur in conducting wound inoculations with pure cultures. In nature the bacteria are spattered from leaf to leaf by rain, and generally penetrate the uninjured leaf surface. It is significant that under such natural conditions *Bacterium tabacum* has not been found infecting any plant except tobacco. Of previous investigators, only two have observed natural infection on plants other than tobacco. Wolf (*12*) observed lesions on cowpeas (*Vigna sinensis* (L.) Endl.) which appeared to start around insect punctures. These lesions remained small, and his conclusion was that the organism was "certainly not actively parasitic." Chapman and Anderson (*2*) observed lesions on tomato leaves which appeared to have started around injuries.

A second line of evidence considered by pathologists in deciding on host relationships concerns the demonstration of the presence or absence of viable pathogenic bacteria in the plant tissues after successful inoculations as judged by the production of disease symptoms. This reisolation and identification of the organism constitutes the concluding steps as prescribed by the Koch postulates.

In these experiments, the organism was reisolated from a variety of plants. Reisolation was most readily accomplished in the greenhouse experiments, since in the field the lesions on plants other than tobacco were soon contaminated by saprophytic bacteria. It was also usually necessary with these other plants to make the reisolations no later than 4 weeks after the inoculations. These reisolations did not always consist of merely removing the bacteria from the point at

which they were originally placed, as, in the case of the bean, the leaf tissue around the point of puncture was killed, and yet the bacteria could be recovered from the inner edge of the chlorotic area. The pathogenicity of the recovered bacteria was readily demonstrated. Evidence of this sort might be accepted as definite proof that the plants concerned were host plants, and, if this point of view were taken, then the list of host plants for *Bacterium tabacum* could be expected to include the greater part of the plant kingdom, since plants nonsusceptible to the toxin are the exception, and the bacteria usually remain alive in the various plant tissues for some time.

As opposed to this point of view, the following arguments may be advanced: (1) The production of typical leaf lesions by wound inoculations with cultures containing toxin cannot be considered as proof of parasitism on the part of the organism, since these lesions were obtained with the toxin whether or not living bacteria were used. (2) The significance of the presence of the bacteria in the tissues of such plants as bean, tomato, and cucumber, after wound inoculation, would appear to depend on further information as to the state of their activity in the tissues of these plants. Thus the bacteria are known to be able to remain alive in limited numbers for a period of months in diseased leaves that are air-dry, and even on such materials as the wood or cloth used in the building of plant beds, and yet it would not be suggested that under these conditions they were active as parasites. It was shown by dilution-plate counts that after wound inoculations the bacteria in the leaf tissues decrease rapidly in all plants except tobacco. Consequently after a few weeks there may be 1,000 times as many bacteria in the tobacco tissues as in bean tissues, for example, and later all the bacteria may be dead in the bean leaves but they still remain abundant in the tobacco. A reasonable conclusion would appear to be that in the dead or weakened tissues that result from the action of the toxin some bacteria are able to live and perhaps secure food and reproduce for a short time, without, however, being parasitic in the sense that they are on tobacco.

The view that parasitic organisms are quite incapable of existing in tissues of plants other than their host plants is further weakened by the results secured by Young (*15*), who found that under favorable artificial conditions many parasitic and saprophytic fungi were able to invade to a limited degree the tissues of numerous plants, but that under normal conditions such infections did not occur. On the basis of his ability to produce infection and to reisolate, he was able to list some 200 new diseases. Johnson (*6*), in similar experiments, was able to demonstrate that *Colletotrichum circinans* (Berk.) Vogl. could invade the tissues of 20 out of 22 species of plants tested, and could be reisolated after surface sterilization, yet so far as known this fungus under natural conditions attacks only species of *Allium*.

Host range is a matter of considerable practical importance because plants listed as hosts are usually regarded as liable to attack by the disease under field conditions, and hence they offer possible means whereby the disease may be carried over winter or be introduced into new areas. It is usually assumed that host plants provide a favorable medium for the multiplication and spread of the parasite. With the exception of tobacco, none of the plants studied showed indications of being liable to infection under natural conditions or of providing favorable conditions for the multiplication and spread of the bacteria;

consequently it is concluded that only species of *Nicotiana* should be regarded as hosts of *Bacterium tabacum*.

SUMMARY

Bacterium tabacum Wolf and Foster, the cause of the wildfire disease of tobacco, secretes a powerful toxin. This toxin passes readily through the ordinary filters, is not precipitated by alcohol or by neutral calcium or lead acetates, is not removed from solution by boiling with bone black, is not affected by formaldehyde, mercuric chloride, or acids, and is thermostable. It is quickly inactivated by dilute alkalies.

When pricked into leaves this toxin destroys the chlorophyll and produces the halo lesions typical of the wildfire disease. The leaves of many plants are susceptible to the action of the toxin, some being much more sensitive than tobacco.

Inoculation experiments with (1) toxin alone, (2) toxin plus bacteria, and (3) bacteria alone yielded large conspicuous lesions from 1 and 2 on a wide range of plant species. With 3 the results were either no lesions or else lesions that remained small and undeveloped in the case of all plants tested, with the exception of species of *Nicotiana*. Tobacco leaves inoculated with toxin-free bacteria developed lesions more slowly than those inoculated with a combination of toxin and bacteria, but the end results were the same.

Poured-plate isolations from many inoculations have shown that in plants other than tobacco the bacteria survive for a few weeks but gradually become fewer and finally die out entirely. In tobacco leaf tissues they continue to live and multiply.

Many plants on which the disease does not usually occur and in the tissues of which the bacteria cannot indefinitely persist may be successfully inoculated as judged by (1) the production of typical wildfire leaf lesions, (2) the ability to reisolate the organism, and (3) positive results from reinoculation. Hence the advisability of unqualified acceptance of the Koch rules of proof is questioned for diseases such as the one under discussion.

It is considered that only species of *Nicotiana* should be regarded as true host plants for *Bacterium tabacum*.

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CHARACTERISTICS OF THE PIROPLASMS BABESIA ARGENTINA AND B. BIGEMINA IN THE UNITED STATES[1]

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PURPOSE OF INVESTIGATION

The purpose of the present investigation was to determine (1) what species of *Babesia* occur in this country, (2) what their morphological and physiological characters are, and (3) whether there is any variation in their reaction to trypan blue, which thus far has been the most promising means of treating clinical cases of babesiasis. In order to ascertain whether *B. argentina* is a valid species, the morphological characters of piroplasms occuring in Europe, Africa, and South America were also studied.

REVIEW OF LITERATURE

VALIDITY OF PROPOSED GENERA AND SPECIES

In 1893 Smith and Kilborne (*13*)[2] stated that the shape of piroplasms resembles that of apple seeds. Subsequent investigators have shown that the piroplasm nucleus is composed of chromatin particles occurring in more than one body; in other words, the chromatin material is scattered. Up to the present time nothing else of morphological importance has been added. Therefore, there appear to be no valid criteria for a number of genera that have been proposed in the family Babesiidae Poche, 1913, and the writer concurs in the views of Wenyon (*15*) and Reichenow (*10*) that only one generic name, *Babesia* Starcovici, 1893, is valid, and that the following names are synonyms: *Piroplasma* Patton, 1895; *Nicollia* Nuttall, 1908; *Nuttallia* França, 1909; *Smithia* França, 1909; *Rossiella* Nuttall, 1912; *Microbabesia* Sohns, 1918; *Babesiella* Mesnil, 1919; and *Françaiella* Yakimoff, 1926. Under these names are reported parasites of bovines, ovines, equines, canines, and rodents.

The occurrence in Argentina of two species of *Babesia*, *B. bigemina* and *B. argentina*, was first reported by Lignières (*5*). In material collected from cases of piroplasmosis in Louisiana, the writer (*8*) noted that the piroplasms were scanty in the peripheral blood; that they were abundant post mortem in the organs, minute in size when compared with other piroplasms known to be *B. bigemina*, and that quadruple infections of erythrocytes were common. Dennis,[3] of the University of California, noted a resemblance to the genus *Nuttallia* and regarded the species as different from *B. bigemina*. Becker,[3] of the Iowa State College of Agriculture, noted that the angle between the longitudinal axes of members of the intraglobular couple was usually about 180°, whereas in *B. bigemina* which he obtained from Texas it was seldom greater than 60°. Therefore, Becker and Rees

[1] Received for publication Nov. 13, 1933; issued May 1934.
[2] Reference is made by number (italic) to Literature Cited, p. 437.
[3] Personal communication.

(*2*) reported the occurrence in Louisiana of a species of *Babesia* (syn., *Piroplasma* Patton, 1895) in addition to the well known *B. bigemina*. This other species, reported by Rees (*8*) as *B. bigemina*, has been determined as *B. argentina* Lignières, 1903.

It appears advisable to recognize *Babesia bigemina* and *B. argentina* as valid species, although Wenyon (*15*), Schilling (*11*), and Reichenow (*10*) do not concur in this view. *B. bovis* Starcovici, 1893, is commonly recognized as a valid species; it is a very small piroplasm like *B. argentina*, and resembles the latter also in that the angle between longitudinal axes of the couple of intraglobular parasites is usually about 180°, but it differs from *B. argentina* in that it is located in the margin of the infected erythrocyte, whereas *B. argentina* is located in the center. As is shown by the data of the present paper, *B. berbera* Sergent and collaborators, 1924, (*12*) resembles *B. argentina*. Yakimoff and collaborators (*16, 17, 18, 19*) have proposed several new species for forms occurring in northern Europe and in Asia that are practically indistinguishable from *B. argentina*.

PIROPLASMS STUDIED BY SMITH AND KILBORNE

The report of Smith and Kilborne (*13*) carries adequate evidence that they were dealing with both *Babesia bigemina* and *B. argentina*. They show in plate 7, figure 2, drawn from a capillary in the kidney, what is clearly a pure infection with *B. argentina;* the parasites are uniform in size, being not more than 2½μ long, which is much too small for *B. bigemina*. In plate 4, figure 4, and plate 6, figures 1 and 2, they show the commonly occurring quadruple infection of erythrocytes which characterizes an infection with *B. argentina*. They report very severe infections and state (*13, p. 61*) that "a long search is necessary before one (parasite) is brought into view", and they say further in the same paragraph: "When present in considerable numbers in the blood the infected corpuscles usually appear in groups in the field of the miscroscope as is shown in the figure referred to (pl. 5, fig. 2), and not uniformly distributed." In further explanation of the figure they state: "The appearance of the infected corpuscles in groups * * * was especially marked in this animal." Lignières (*5*) pointed out that the latter phenomenon is a characteristic of infections produced by *B. argentina* but not of infections produced by *B. bigemina*. Although it has been copied in text books as a figure of *B. bigemina*, this illustration of Smith and Kilborne (pl 5, fig. 2) is clearly a figure of *B. argentina*. The following statement occurs in their paper (*13, p. 61*):

> With only 1 or 2 per cent, or even 10 per cent, of infected corpuscles in the circulating fluid, it would be difficult to account for the enormous daily losses of blood corpuscles in the acute fever. The difficulty is cleared up by sacrificing an animal in the earlier days of the fever and examining the internal organs for infected corpuscles. Large numbers of parasites are found within corpuscles in the capillary blood of congested areas, such as those of the heart muscle and of the omentum.

The foregoing citations are good descriptions of a typical infection with *Babesia argentina*, but not of one with *B. bigemina*.

Intraglobular forms of the parasite are illustrated in Smith and Kilborne's paper (*13*) in text figure 3, which shows that the angles between the longitudinal axes of the couples are about 150°, 160°, and 180°, respectively; the organisms figured are *Babesia argentina*.

On the other hand, in plate 5, figure 3, a smear of peripheral blood, the organisms are *B. bigemina;* a solitary spindle-shaped form is about 7μ long, which is longer than the diameter of the normal bovine corpuscle, whereas the other parasites in grouped couples are 4μ long. The unstained parasites figured in plate 8, figures 1–5, are *B. bigemina.* Though other pertinent citations might be furnished, the data presented by Smith and Kilborne show adequately that two species of *Babesia, B. bigemina* and *B. argentina,* were seen and described by them under the name *Pyrosoma bigeminum.*

EFFECT OF TRYPAN BLUE ON PIROPLASMS

A number of prominent veterinarians who have large cattle practices in Louisiana have told the writer they they do not use trypan blue in cases of bovine piroplasmosis. These practitioners stated that whereas in certain outbreaks clinical cases were cured by intravenous injections of the drug, in other outbreaks it had no apparent effect.

Trypan blue was found by Nuttall and Hadwen (*7*) and by Theiler (*14*) to be a specific in infections with *Babesia canis* (Piana and Galli-Va'lerio, 1895) and *B. bigemina,* but was found by Brumpt (*3*) to have no marked therapeutic action in infections with *B. argentina.*

MATERIAL

The material used in the present investigation was obtained from the following sources: (1) Smears from the heart of a cow that was in a herd of purebred Holstein-Friesians, at Lafayette, La., in which 12 out of about 13 cases of infection with *Babesia argentina* proved fatal; (2) a strain of this species of *Babesia* obtained by infecting another cow at the Jeanerette station with the progeny of a tick, *Boophilus annulatus* (Say, 1821), Curtice, 1891, taken from one of these cows; (3) 35 other cases of infection with *B. argentina,* all occurring in Louisiana; (4) 8 cases of infection with a strain of *B. bigemina* shipped to Jeanerette, La., in blood from Texas; (5) 2 naturally occurring Louisiana cases involving *B. bigemina;* and (6) smears of the following piroplasms which were sent to the writer through the courtesy of the investigators named: *Babesia bovis* from Dr. R. Wetzel of the Tierärztliche Hochschule, Hannover, Germany; *Babesiella berbera* from Dr. Sergent, of the Institut Pasteur d'Algérie; *Babesiella minor* Quevedo, 1918, and *Babesia bigemina* from Dr. F. Rosenbusch, of Buenos Aires, Argentina. The writer believes that all these forms belong in the genus *Babesia* and considers *Babesiella minor* a synonym of *Babesia argentina.*

MORPHOLOGICAL CHARACTERS OF THE PIROPLASMS

BIOMETRY

Table 1 shows that *Babesia bigemina* is longer than *B. argentina,* the latter being about the same length as *B. berbera,* and, furthermore, that *B. bovis* is the smallest of the four species. The various forms of *Babesia* occurring in the peripheral blood have mean lengths that vary from 2.26 $\pm$ 0.04μ (smear of *B. bigemina* from Argentina) to 5.00 $\pm$ 0.05μ for *B. bovis.* There were three length groups, about 4, 3, and 2.25μ, respectively, with *B. bigemina* in the first group, *B. argentina* and *B. berbera* in the second, and *B. bovis* in the third. The ranges in length (table 1) were from 2.5 to 5.5μ for *B. bigemina,* 2 to 4.5μ for *B. argentina,* 2 to 5μ for *B. berbera,* and 2 to 3μ for *B. bovis,* so that,

except for *B. bovis*, there was almost complete overlapping. In smears of *B. bigemina* from Argentina the parasites were longer than in smears of the same species from Texas, the means being $5.00 \pm 0.05\mu$ and $4.02 \pm 0.04\mu$. This fact supports the statements which are found generally in the literature that discontinuous variations occur between different strains in the same species.

Table 1 shows that *B. bigemina* occurring in smears made from the heart is smaller than in those made from the peripheral blood, the mean lengths being $4.02 \pm 0.04\mu$ and $3.70 \pm 0.04\mu$; this applies also to *B. argentina*, with corresponding lengths of $3.14 \pm 0.04\mu$ and $2.75 \pm 0.03\mu$. However, in each species these differences of less than 1μ were not so great as those shown in the drawings by Smith and Kilborne (*13*) all of which were supposed to be of *B. bigemina*.

TABLE 1.—*Length of specimens of Babesia and magnitude of angle of the intraglobular couple*

Kind of smear and source	Length		Magnitude of angle	
	Microns	Frequency	Degrees	Frequency
BABESIA BIGEMINA				
Peripheral blood (Texas)	2.5	8	20	40
	3.0	18	30	34
	3.5	33	45	13
	4.0	34	60	12
	4.5	17	90	37
	5.0	38	120	4
	5.5	2	150	6
			180	4
Mean or total	4.02±0.04	150	57.0	150
Heart blood (Texas)	2.5	12	20	12
	3.0	11	30	35
	3.5	21	45	13
	4.0	41	60	8
	4.5	10	90	26
	5.0	5	150	3
			180	3
Mean or total	3.70±0.04	100	56.8	100
Peripheral blood (Argentina)	4.5	6	20	14
	5.0	13	30	5
	5.5	6	45	2
			60	2
			90	2
Mean or total	5.00±0.05	25	32.8	25
BABESIA ARGENTINA				
Peripheral blood (Louisiana)	2.0	3	20	8
	2.5	23	30	9
	3.0	31	45	6
	3.5	31	60	6
	4.0	10	90	21
	4.5	2	120	7
			150	18
			180	25
Mean or total	3.14±0.04	100	109.9	100
Heart blood (Louisiana)	2.0	9	20	3
	2.5	44	30	4
	3.0	36	45	6
	3.5	9	60	7
	4.0	2	90	14
			120	7
			150	20
			180	39
Mean or total	2.75±0.03	100	129.9	100

TABLE 1.—*Length of specimens of Babesia and magnitude of angle of the intraglobular couple*—Continued

BABESIA ARGENTINA (SYN., BABESIELLA MINOR)

Kind of smear and source	Length		Magnitude of angle	
	Microns	Frequency	Degrees	Frequency
Peripheral blood (Argentina)	2.0	3	20	1
	2.5	8	30	3
	3.0	10	90	6
	3.5	3	120	1
	4.0	1	150	7
			180	7
Mean or total	2.82±0.07	25	123.2	25

BABESIA BERBERA (SYN., BABESIELLA BERBERA)

Peripheral blood (Algeria)	2.0	9	20	6
	2.5	14	30	9
	3.0	15	45	1
	3.5	6	60	3
	4.0	4	90	13
	4.5	1	120	6
	5.0	1	180	12
Mean or total	2.89±0.06	50	93.3	50

BABESIA BOVIS

Peripheral blood (Germany)	2.0	13	20	1
	2.5	11	30	1
	3.0	1	45	2
			90	4
			120	1
			150	3
			180	13
Mean or total	2.26±0.04	25	136.4	25

With respect to the angle occurring between the longitudinal axes of the members of the couple in piroplasms, the writer's data show two main groups as follows: (1) *B. bigemina*, with a mean angle of 57° in the Texas strain and 32.8° in the Argentine strain; and (2) *B. argentina* from Louisiana and Argentina, *B. berbera* (syn., *Babesiella berbera*), and *Babesia bovis*, in which species the range of the mean angle was from 93.3° for *B. berbera* to 136.4° for *B. bovis*. It will be noted from table 1 that for all the species in the second group, except *B. berbera*, the mode was about 180°. The latter species was bimodal, i.e., at 90° and 180°. A difference of 20° between the means of *B. argentina* in the peripheral blood and in the heart blood shows that in a given species this character is subject to wide variations. In living couples of *B. argentina*, the writer has observed rapid rotations from an angle of 90° to one of 270°. In fixed material, the maximum figure that can be read is 180° and, therefore, the statistics may not show the real differences between the angles of the two species. Table 1 shows that in the four species of piroplasms the range of variation was from 20° to 180°, i.e., there is complete overlapping. Although in the smaller *Babesia*, i.e., *B. argentina*, *B. berbera*, and *B. bovis*, the mean angle is greater than in *B. bigemina*, the limits of variation in all four species are the same so far as the angle is concerned.

CYTOLOGY

Drawings, all on the same scale, of *Babesia bigemina* from Texas, *B. argentina* from Louisiana and Argentina, *B. berbera* from Algeria, and *B. bovis* from Germany are presented in figure 1. These drawings

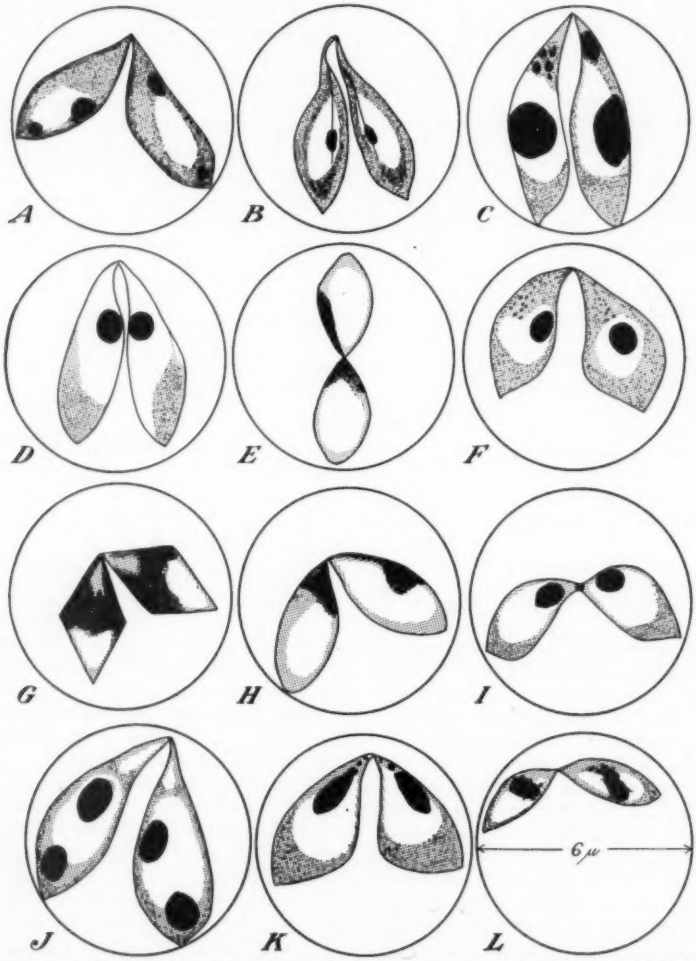

FIGURE 1.—Camera lucida drawings of four species of *Babesia: A, B, C, B. bigemina* from peripheral blood; *D, B. bigemina* from heart blood; *E, F, B. argentina* from peripheral blood; *G, H, B. argentina* from heart blood; *I, B. argentina* (syn., *Babesiella minor*) from peripheral blood; *J, K, Babesia berbera* (syn., *Babesiella berbera*) from peripheral blood; *L, Babesia bovis* from peripheral blood. *B*, from a preparation stained with iron haematoxylin; all others with Giemsa's stain.

show in general the same features as those of previous investigators; there are no morphological criteria other than size, magnitude of angle, and position within the erythrocyte for the determination of species.

Typical forms of *Babesia bigemina* are shown in figure 1, *A*, *B*, and *C*. Figure 1, *B*, from a preparation which was stained in iron haematoxylin, shows a fine fibril such as was described by Dennis (*4*) as the rhizoplast. It could not be found in preparations in which other stains were used. The writer was unable to differentiate the structure which Dennis (*4*) described as the nuclear membrane. However, there was usually a halo about the large chromatin granule.

Figure 1, *D*, illustrating the appearance of *Babesia bigemina* from the heart, shows 1 mass of chromatin in the nucleus, not 2 or 3 masses such as occur in parasites from peripheral blood; this characteristic was general in the writer's material.

Figure 1, *E* to *H*, illustrates *Babesia argentina* from Louisiana, *E* and *F* being from peripheral blood and *G* and *H* from heart blood. *G* is similar to the couple of spindle-shaped parasites shown in text figure 3 of Smith and Kilborne's (*13*) paper. These authors mentioned spindle forms as being typical of *B. bigemina*. The writer has found spindle forms consistently in *B. argentina* but never in *B. bigemina*.

I, in figure 1, is *Babesia argentina* (syn., *Babesiella minor*) from Argentina. *J* and *K* are *B. berbera* (syn., *Babesiella berbera*) from a preparation by the Institut Pasteur d' Algérie. The size of *B. berbera* in *J* is similar to that of *B. bigemina;* although large parasites were rare in *B. berbera*, their occurrence was demonstrated by statistics also. *B. berbera* (*K*) is indistinguishable from *B. argentina* (*F*). Figure 1, *L*, shows that *B. bovis*, which was received from Hannover, Germany, was easily distinguishable from the three other species both by its smaller size and by its marginal position within the erythrocyte.

PHYSIOLOGICAL CHARACTERS OF THE PIROPLASMS

NUMBER DURING LIFE IN THE PERIPHERAL BLOOD AND POST MORTEM IN THE ORGANS

In the writer's cases of *Babesia argentina* infection, the parasites were scanty in the peripheral blood, agreeing in this respect with descriptions of infection produced by this species in other parts of the world. On September 18, 1931, blood was drawn from a "carrier" at Kaplan, La., defibrinated, and then injected intravenously into two Brahman-Hereford cows, nos. 77 and 78, at Jeanerette, La. Both cows reacted on the sixth day, no. 77 died on the eleventh day and no. 78 on the twelfth day; each case had haemoglobinuria for 3 days prior to death. During this time infected corpuscles were scanty and hard to demonstrate in the smears. In occasional smears, however, they were plentiful; when plentiful they occurred in groups. Figure 2 shows the occurrence of *B. argentina* in 15 out of 16 corpuscles in the blood of cow 77.

It has been stated elsewhere in this paper that whereas *Babesia argentina* was scanty in the peripheral blood it occurred abundantly in smears made from the organs. Figure 3 represents 16 corpuscles in a capillary of the brain of cow 77; 15 of the corpuscles were parasitized; in one case there was a quadruple infection, in another a triple infection, and there was one couple of extraglobular piroplasms.

In *Babesia bigemina* infections the parasites were numerous in the peripheral blood and were uniformly distributed, never occurring in groups as in the case of infection produced by *B. argentina*. Capillaries which were congested with infected erythrocytes were not found

post mortem in the organs, not even in the brain. This was in marked contrast to the condition occurring in fatal cases produced by *B. argentina*.

The foregoing data indicate the following things: (1) The principal seat of multiplication of *Babesia argentina* is in the capillaries, par-

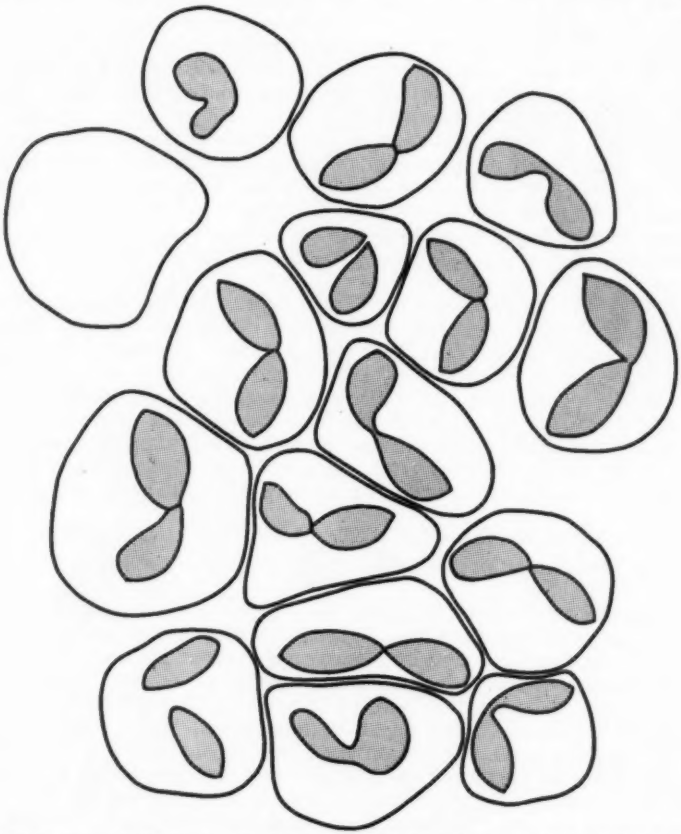

FIGURE 2.—Camera lucida drawings showing the occurrence of *Babesia argentina* in 15 out of 16 corpuscles of the peripheral blood; live blood from cow 77.

ticularly in those of the internal organs, and when released into the peripheral circulation *B. argentina* occurs in clusters and is not uniformly distributed; (2) infections with *B. bigemina* are heavy in the peripheral blood; (3) in the peripheral blood *B. bigemina* does not occur in groups as does *B. argentina;* (4) the infections with *B. bigemina* in the capillaries of the internal organs are light as compared with those of *B. argentina*.

BEHAVIOR IN CULTURE

By the method of Bass and Johns (*1*) the writer was able to cultivate *Babesia argentina* for 96 hours, but he did not succeed in

establishing subcultures. *B. bigemina* could not be cultivated, the parasites becoming rounded up within several hours after transfer to the culture tubes and disappearing within 36 hours.

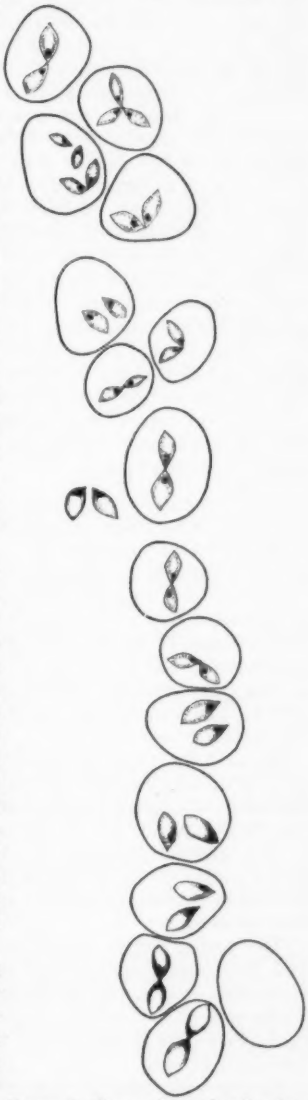

FIGURE 3.—Camera lucida drawings from a capillary in the brain of cow 77, showing 16 corpuscles, 15 of which were parasitized with *Babesia argentina;* also 1 pair of extraglobular corpuscles.

REACTIONS TO TRYPAN BLUE

A case of infection with *Babesia bigemina* was discovered in bull 70 on April 2, 1932, at the experimental barn. Rees (*9*) has reported this case as one of accidental transmission by an infected lancet. A count on April 2 showed 120 infected erythrocytes per 1,000. This bull weighed 650 pounds and was treated by intravenous injection with 600 mg of trypan blue in 500 cc of physiological saline. In smears which were taken 4 hours after treatment there was only an occasional parasite, and on the following morning there were none. The prognosis on the morning of April 4 was favorable, but the bull was accidently killed by a drenching procedure. Smears from the heart, brain, spleen, and liver were all negative for piroplasms.

Bull 71 had a very severe case of infection with *Babesia bigemina* on August 2, 1932, the urine being colored a deep blood red, and although no counts were made, the infection in the peripheral blood was heavy. This bull weighed about 800 pounds. It was injected with 1 g of trypan blue at 4 p.m. by the same method as with bull 70. At 10 p.m. there were very few parasites in the smears and these appeared to be poorly defined and did not stain as do normal piroplasms. The smears that were made the next morning were negative. Within 24 hours after treatment haemoglobinuria disappeared; the bull made a rapid recovery.

The writer has been favored with the cooperation of H. Laughlin, a veterinarian, in treating nine cases of infection with *Babesia argentina* that occurred in his local practice from March 2 to August 31, 1932, microscopic diagnoses having been made by the writer. Details concerning two of these cases were as follows:

A purebred Jersey bull weighing about 1,500 pounds had haemoglobinuria and a temperature of 107° F. on July 13, 1932. Five smears showed *Babesia argentina;* in one smear the

parasites were fairly numerous. At 9 a.m. the bull was injected intravenously with about 2 g of trypan blue; at 9 p.m. it was in extremis. At 5:30 a.m. on July 14, it was dead. While the body temperature was still near normal, smears of the heart, brain, and spleen were made, and these showed very heavy infections with *Babesia argentina*. The organisms were apparently rounded up but stained normally.

A grade Jersey steer weighing about 600 pounds was observed on August 31, 1932, at 11 a.m. and showed the same symptoms and parasites as the Jersey bull. It was injected intravenously at 12 m. with 3 g of quinine hydrochloride in 500 cc of normal saline, and at 1 p.m. with 1 g of trypan blue. On September 1, its condition was unchanged. On September 2, at 8 a.m., it was dead. Necropsy results were the same as in the case of the Jersey bull.

All seven of the remaining cases of *Babesia argentina* were treated with trypan blue only. One was examined post mortem on August 27, about 1 hour after death. This case had received two intravenous injections of trypan blue, one 72 hours before death and the other 48 hours before death. Smears of the organs showed numerous parasites apparently normal when compared with those in untreated fatal cases. Of the other six cases, not examined post mortem, three died within 24 hours, another within 48 hours, and the remaining two within 96 hours after treatment.

SUMMARY

A species of *Babesia* occurring in the United States has the following characters: (1) Minute size, (2) scanty occurrence in the peripheral blood although occasional occurrence in the field of the microscope in groups, (3) heavy infection in the internal organs, where quadruple infections of erythrocytes are common, and (4) the common occurrence of an angle of about 180° between the longitudinal axes of the two members of the intraglobular couple. On the basis of the above characters this species has been determined as *Babesia argentina* Lignières, 1903. The author's investigation has confirmed the above-mentioned characteristics.

Babesia bigemina is larger than *B. argentina*, infections are heavy in the peripheral blood and scanty in the internal organs, and the angle between the longitudinal axes of the members of the intraglobular couple is usually less than 60°.

The present investigations indicate that the piroplasms described and illustrated by Smith and Kilborne in 1893 included *B. argentina* as well as *B. bigemina*, although these workers did not differentiate the two species.

The mean length of a Texas strain of *B. bigemina* was $4.02 \pm 0.04\mu$ and that of an Argentine strain was $5.00 \pm 0.05\mu$; the mean lengths of a Louisiana strain and an Argentine strain of *B. argentina* were $3.14 \pm 0.04\mu$ and $2.82 \pm 0.07\mu$, respectively; that of an Algerian strain of *B. berbera* was $2.89 \pm 0.06\mu$; and that of a German strain of *B. bovis* was $2.26 \pm 0.04\mu$.

B. bigemina and *B. argentina* in the peripheral blood were larger than in the heart blood, but the difference in each case was smaller than 1μ, and less than that shown between peripheral blood forms and heart forms in the figures of Smith and Kilborne.

The magnitude of the mean angle formed by the intraglobular couple in piroplasms was as follows: (1) Argentine strain of *B. bigemina*

32.8°, (2) Texas strain of *B. bigemina*, 57°, (3) Argentine strain of *B. argentina* (syn., *Babesiella minor*), 123.2°, (4) Louisiana strain of *B. argentina*, 109.9°, (5) Algerian strain of *B. berbera*, 93.3°, and (6) German strain of *B. bovis*, 136.4°.

The writer's drawings of *B. bigemina* show this piroplasm in the heart blood with only one mass of chromatin but in the peripheral blood with more than one mass.

Spindle-shaped forms have been shown in the present paper to characterize *B. argentina* and not *B. bigemina*, contrary to the findings of Smith and Kilborne (*13*).

No significant morphological differences were detected between *B. argentina* and *B. berbera*, nor between a Louisiana strain and an Argentine strain of *B. argentina* (syn., *Babesiella minor*), but *B. bovis* was distinguishable from both of the above-named species by its smaller size and its marginal position within the erythrocyte.

B. argentina was cultivated for 96 hours in vitro; *B. bigemina* could not be cultivated.

In agreement with the results of previous investigators it was found that *B. bigemina* was killed by intravenous injections of trypan blue, but *B. argentina* was not demonstrably affected.

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THE CALCIFYING PROPERTIES OF GREEN, ARTIFICIALLY DRIED, AND SUN-CURED PASTURE HERBAGE[1]

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INTRODUCTION AND REVIEW OF PREVIOUS INVESTIGATIONS

The potency of the antirachitic vitamin in green forage has been a subject of considerable controversy. The difference of opinion regarding the antirachitic or the calcifying properties of green feeds is probably due to the difficulty in demonstrating through biological tests the small amounts of such properties contained in green feeds. Little experimental work has been done to determine the calcifying properties of pasture herbage, although considerable work has been done on other classes of green materials used in human consumption.

Chick and Roscoe (*3*)[3] demonstrated a slight but definite antirachitic value in summer-grown spinach, but failed to observe any antirachitic value in spinach grown in the open during winter, spring, or fall. Later Roscoe (*11*) reported a slight effect upon calcification in rats produced by the addition of 10 percent of green summer-grown spinach leaves to the experimental ration. Shipley, Kinney, and McCollum (*15*) found that the ether extract of 250 g of alfalfa leaves or clover blossoms, when mixed with a kilogram of the basal ration, caused healing of rickets in rats. However, they were unable to note an antirachitic action of ether extracts of similar amounts of dry spinach, brussels sprouts, celery, or cabbage. Bethke, Kennard, and Kick (*1*) found that leg weakness in chickens was not prevented by incorporating 18 percent (dry-matter basis) of fresh green red clover in the basal ration.

Research by Russell (*12*) showed that artificially dried alfalfa leaves possessed only a small amount of vitamin D, but that alfalfa dried in the sun was higher in its antirachitic action. This finding was confirmed by Steenbock and his coworkers (*17*), who concluded that the antirachitic properties of hays are related to their exposure to sunlight. Recently Smith and Briggs (*16*) presented further evidence to show that the antirachitic value of alfalfa was dependent upon its exposure to sunshine. The alfalfa that was cured in the absence of sunlight was deficient in the antirachitic factor.

In studies with dairy cattle, Hart and his coworkers (*6, 8*) concluded that there was apparently enough of the antirachitic factor present in a ration made up of 40 pounds of green grass, 30 pounds of silage, and 14 pounds of grain when fed to cows producing from 40 to 60 pounds of milk per day, to maintain calcium equilibrium provided

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[2] The writers are indebted to Harvey Murer and Harold Gerritz, who were responsible for the analytical work, and to M. S. Grunder, who supervised the artificial drying of the herbage.

[3] Reference is made by number (italic) to Literature Cited, p. 445.

the calcium and phosphorus intake was sufficiently high. Calcium equilibrium was established when CaO was fed at a rate of approximately 1.5 percent of the daily ration. This was the case whether the animals were maintained out of doors or in stables without direct sunlight. Hart and others (*7*) failed to produce favorable calcium assimilation in heavy-producing cows that were given irradiated yeast, potent in vitamin D. They therefore concluded, as reported by Clark (*5*), that the ability of fresh green grass to cause calcium assimilation in milking cows was probably due to some factor other than its vitamin D content. Newlander and Jones (*10*) reported that cows receiving a ration of timothy hay, corn silage, and grain utilized calcium and phosphorus as well as when grass, either green or artificially dried, was added to the ration.

Results reported by Luce (*9*) show that cows receiving a diet of fresh green-pasture grass produced milk of higher antirachitic or growth-promoting properties, even when they were maintained indoors in the absence of direct sunlight, than cows not receiving green grass in the ration. Likewise, Chick and Roscoe (*4*) found some evidence that the inclusion of fresh green feed in the ration caused an increase in the vitamin D content of milk.

It was the purpose of this investigation to measure the comparative calcifying properties of pasture herbage when fed in a green, artificially dried, and sun-cured condition.

EXPERIMENTAL PROCEDURE

The preventive method of procedure used in determining the antirachitic or calcifying properties of the pasture herbage was essentially that outlined by Sherman and Stiebeling (*13*, *14*), in which the basal ration is adequate in other respects but devoid of vitamin D. Young rats 21 to 24 days of age receiving the Sherman and Stiebeling (*13*) vitamin-D-deficient test diet and weighing from 45 to 65 g were used in this investigation. They were placed on experiment when approximately 28 days of age. The animals used were from the California Institute of Experimental Biology, pied strain, reared from mothers whose diet consisted of ground whole wheat, 67.5 percent; casein, 15 percent; whole-milk powder, 10 percent; milk fat, 5 percent; sodium chloride, 1 percent; and calcium carbonate, 1 percent. Fresh lettuce was fed twice each week.

During the experiment the rats were kept in individual, all-metal cages with raised screen floors and maintained in a darkened room devoid of all natural light. The basal diet consisted of cornstarch, 66 percent; extracted casein, 18 percent; dried brewers' yeast, 10 percent; Osborn and Mendel salt mixture, 4 percent; c.p. sodium chloride, 1 percent; and dried spinach, 1 percent. In those instances in which rats were receiving pasture herbage as a part of their ration the spinach, which supplied vitamin A, was eliminated and the percentage of cornstarch correspondingly increased. The rats were allowed free access to their feed at all times, and clean fresh water was always before them. Individual live weights were recorded each week and feed consumption was checked daily.

In comparing the calcifying properties of pasture herbage fed in the different ways, two experiments were conducted. The first test was a comparison of the calcifying properties of green with artificially

dried pasture herbage; the second compared artificially dried with sun-cured herbage. In each test negative and positive control groups were maintained. The negative control groups received the basal ration only, whereas the positive control groups received, in addition to the basal ration, 4 drops of 250 D viosterol per week. The viosterol was fed directly into the mouths of the rats.

The material being tested was fed at 3-percent, 6-percent, and 9-percent levels. The percentage of herbage in the experimental rations was calculated on a dry-matter basis.

Insofar as possible the animals were assigned to control and test groups so that litter mates and initial live weight would be equally distributed among the various lots. In the first experiment an equal distribution of sex was maintained in each group. In the later experiment, only male rats were used. It was found necessary for reasons not attributed to this investigation to discontinue the first experiment at the end of the seventh week. Therefore, the mean ash analyses of the first experiment are not on a strictly comparable age basis with those of the second experiment, which terminated at the end of the eighth week. This should not, however, interfere with the interpretation of the results, for a comparison of improvement in the test animals over their respective negative controls, relative to the improvement of the positive over the negative controls, is used as the basis of interpretation.

In outlining the procedure of this method of quantitative determination of vitamin D, Sherman and Stiebeling (*14, pp. 689, 692*) make the following statement:

> It will be noted that our basal diet is radically different from the rickets-producing diets commonly used for vitamin D studies which involve the use of the line test. None of our rats receiving only the basal diet here used developed rickets as judged by the line test, although occasionally a few beaded ribs were observed in rats 80 days or more in age. We are not dealing with rickets as ordinarily understood.
>
> We consider to have equivalent vitamin-D content those amounts of materials under investigation which induce a degree of calcification midway between the minimum values fixed by the diet without added vitamin D and the maximum values obtained with an abundance of supplementary vitamin D. It should be emphasized that this method is feasible only when the groups of animals used for testing vitamin D can be compared with two control groups each containing representatives from the same litters and matched in sex and weight, one receiving no added vitamin D, the other receiving a fixed liberal supply.

At the termination of the experimental periods the rats were chloroformed and the femur bones dissected out, carefully cleaned of all adhering tissue, weighed, dried, extracted with alcohol, reweighed, and ashed. The percentage of ash in the fresh femur bone was used as a criterion of the degree of calcification.

The pasture herbage used in this study was a mixture of English ryegrass (*Lolium perenne*), Italian ryegrass (*L. italicum*), and a small percentage of white clover (*Trifolium repens*). The pasture was irrigated with a sprinkler at weekly intervals to insure uniform rate of growth throughout the season. The samples of herbage used, with the exception of that fed green, were collected once each week, at 1 p.m., and represented herbage that was 3 weeks old.

The 3-weeks-old grass that was fed in a green condition was cut daily at 1 p.m., mixed, and ground into a fine mash. This was thoroughly mixed with the basal ration at the desired percentage for

each rat. The resultant mixture was a dry, sticky mash that proved to be quite palatable to the animals.

The samples that were to be artificially dried were taken to the drier immediately after being cut. Drying was accomplished by means of a direct heat rotary drier. The material passed through the machine in about 10 minutes and was dried at an outlet temperature of 165° to 175° C.

The herbage to be sun-cured was exposed on a canvas to the direct rays of the sun and was withheld from any leaching that might have resulted from rain or dew. The exposure to direct sun's rays of varying intensity during the drying process averaged about 15 hours. The exposure of each portion of the sun-dried grass took place over a 2-day period.

In all cases the herbage was thoroughly mixed, finely ground, and remixed prior to its incorporation into the experimental rations. The ration containing as much as 9 percent of grass (dry-matter basis) was readily eaten by the experimental animals.

RESULTS

Table 1 gives a summary of the first experiment and shows the effect upon growth and calcification in rats of adding graded portions of green and artificially dried pasture herbage to the basal diet. Table 1 also summarizes the records of the second test and shows the effect upon growth and calcification of adding graded portions of dehydrated and sun-cured herbage to the basal diet.

In interpreting the antirachitic potency of materials studied by the preventive method of procedure, investigators have measured results in the following ways: (1) Percentage of ash in the green femur bone (2) percentage of ash in the dried extracted femur bone, and (3) the ratio of the ash to the organic residue of the femur bone.

In this investigation the average of 150 observations of rat bones in which the percentage of ash in the green femur bone was correlated with the percentage of ash in the same bone after it had been dried and extracted revealed a coefficient of 0.880 ± 0.039. The correlation between the percentage of ash in the green femur bone and the ash-organic-residue ratio was 0.743 ± 0.025. These high degrees of correlation illustrate the possibility of using any one of the three methods as a criterion of the degree of calcification in the bones of rats. The method of basing the results upon the percentage of ash in the green femur bone has the advantage of being less time consuming and less expensive.

The data on mean percentage of bone ash and gain in live weight show smaller differences between the negative and positive control groups in the first experiment than in the second. However, in each experiment the range of difference between the negative and positive control group was statistically significant.

In this connection, Sherman and Stiebeling (*13, pp. 500–501*) state:

> Although we have encountered wide seasonal and litter variations in the level of calcification found in both negative and positive controls, we find that this does not interfere with our interpretation of results when we use as a basis of comparison the improvement in test animals over their respective negative controls, relative to the improvement of the positive over the negative controls.

TABLE 1.—*Effect upon growth and calcification of adding graded portions of green, artificially dried, and sun-cured pasture herbage to the basal diet of rats, for experimental periods of 49 and 56 days*

FIRST EXPERIMENT (GREEN AND ARTIFICIALLY DRIED GRASS COMPARED), 49 DAYS

Group	Quantity of pasture herbage (dry-matter basis) added to the basal diet	Cases	Average initial live weight	Average gain in live weight	Average weight of green femur bone	Ash in green femur bone		Improvement over negative controls		
								Percent	Critical ratio [a]	Relative to positive controls
	Percent	*Number*	*Grams*	*Grams*	*Grams*	*Grams*	*Percent*			*Percent*
Negative controls, fed basal diet only		10	63.0±0.59	140.0±1.42	0.5945±0.081	0.2198±0.056	35.60±0.22			
Groups receiving green grass in addition to the basal diet	3	9	63.9± .53	151.2±1.65	.6115± .051	.2400± .034	38.78± .32	3.18	8	82
	6	10	65.0± .67	142.0±1.44	.6045± .081	.2360± .060	38.60± .23	3.00	9	77
	9	10	64.0± .48	148.0±1.22	.6045± .045	.2400± .047	39.80± .36	4.20	10	108
Groups receiving artificially dried grass in addition to the basal diet	3	10	66.0± .47	136.0±1.54	.5845± .077	.2280± .039	38.80± .28	3.20	9	82
	6	8	65.0± .65	137.5±1.40	.6065± .092	.2375± .068	38.75± .19	3.15	11	81
	9	10	63.0± .59	142.0±1.24	.5945± .054	.2380± .044	39.20± .28	3.60	10	92
Positive controls, given viosterol in addition to the basal diet		8	63.8± .70	145.0±1.03	.5945± .051	.2325± .037	39.50± .28	3.90	11	100

SECOND EXPERIMENT (ARTIFICIALLY DRIED AND SUN-CURED GRASS COMPARED), 56 DAYS

Negative controls, fed basal diet only		10	68.0±1.00	138.0±1.24	.5945± .045	.1860± .065	30.60± .43			
Groups receiving artificially dried grass in addition to the basal diet	3	9	65.0± .67	237.8± .88	.7555± .022	.3022± .024	39.12± .26	8.52	17	89
	6	9	67.2± .44	220.0±1.16	.7445± .040	.2888± .030	38.66± .26	8.06	16	84
	9	9	67.2± .65	240.0± .95	.7445± .071	.2956± .047	40.00± .30	9.40	18	98
Groups receiving sun-dried grass in addition to the basal diet	3	10	67.0± .59	226.0±1.28	.7345± .047	.2880± .051	38.40± .27	7.80	15	81
	6	10	66.0± .47	226.0±2.26	.7245± .079	.2820± .067	39.00± .07	8.40	10	88
	9	9	62.8± .45	248.8±2.08	.7995± .059	.3156± .058	39.56± .25	8.96	18	93
Positive controls, given viosterol in addition to the basal diet		10	66.0± .77	238.0±1.73	.7745± .068	.3100± .054	40.20± .21	9.60	20	100

[a] Critical ratio indicates ratio of the difference to its probable error.

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Had it been possible to continue the first experiment through the eighth week, the differences noted probably would have been less pronounced. The fact that this experiment was conducted during midsummer may also have been a contributing factor, although the test animals were cared for in a room devoid of sunlight or other natural light.

Animals receiving additions of green, or artificially dried, or sun-cured pasture grass in the basal diet all showed significantly higher calcification than did the negative controls. The mean percentage of bone ash for each group, except those receiving 9 percent of green grass as a supplement, fell within the range of differences existing between the negative and positive controls.

When either green or artificially dried pasture herbage was fed as 3 percent of the dry matter of the ration, there was an improvement in the percentage of bone ash, which amounted to 3.18 percent and 3.20 percent, respectively. In the second test, when either the artificially dried or the sun-cured herbage was fed at the 3-percent level, the increases over the negative controls were 8.52 percent and 7.80 percent, respectively. Further additions of 6 and 9 percent of the various supplements to the diet did not materially increase the degree of calcification. Apparently there was sufficient of the calcifying factor in the green grass, artificially dried grass, or sun-cured grass when fed as 3 percent of the ration to cause good calcification in the experimental animals. Whether this marked improvement in calcium deposition was due entirely to vitamin D, or, as Hart and his coworkers (reported by Clark (*5*)) have suggested, was partly due to other factors contained in green plant tissue, remains unsolved. In any event it is apparent that the potency was relatively the same in the green, the artificially dried, and the sun-cured herbage used in this investigation. At each of the three levels of feeding, the artificially dried herbage was relatively as efficient in furnishing the calcifying factor as was the green material. Exposure of the herbage to high temperature, therefore, had no deleterious effect upon the factor causing calcification in rats.

Samples of pasture herbage that were exposed to 15 hours of direct sunlight were not appreciably more efficient in promoting calcification than was similar herbage that had been artificially dried. Russell (*12*) found alfalfa to be higher in its antirachitic potency when sun-cured than when machine-dried or air-dried in the absence of sunlight. Smith and Briggs (*16*) reported that alfalfa that had been exposed to Arizona sunlight for 15 hours and 5 minutes possessed only mild calcifying properties. In their assays these workers used the line test with a high-calcium, low-phosphorus diet.

According to Bethke, Kick, and Wilder (*2*) the ratio of calcium to phosphorus in the ration produces a marked effect upon the growth and calcification in rats. They state that the most favorable calcium-phosphorus ratio for growth and bone development was between 2.00 and 1.00 of calcium to 1.00 of phosphorus, and that the vitamin D requirements were higher when the ratio was widened beyond these limits. Table 2 gives the percentage of calcium and phosphorus and the calcium-phosphorus ratio for each of the rations used in the second experiment of this investigation. It will be noted that these rations contained considerably more phosphorus, and therefore probably had a more favorable ratio, than the diets used by

Russell (*12*) or by Smith and Briggs (*16*). The calcium-phosphorus ratios of the experimental rations used in this investigation differed so little that differences in the degree of calcification could not be attributed to differences in this ratio.

TABLE 2.—*The calcium and phosphorus content and the ratios of calcium to phosphorus in the rations of the second experiment*

Ration	Calcium	Phosphorus	Ca/P
	Percent	*Percent*	
Basal diet	0.684	0.705	0.97
Basal mixture with the addition of—			
3 percent of artificially dried grass	.668	.705	.95
6 percent of artificially dried grass	.663	.680	.98
9 percent of artificially dried grass	.663	.659	1.00
3 percent of sun-dried grass	.634	.692	.92
6 percent of sun-dried grass	.666	.673	.99
9 percent of sun-dried grass	.660	.665	.99

Evidence is herein presented which confirms the belief that when the calcium and phosphorus intake of the animal is approximately normal, green plant tissue is a contributing factor in causing calcium assimilation in the animal's body. It also appears that this property of green plant tissue is not destroyed by artificial dehydration as employed in this investigation.

CONCLUSIONS

In this paper data are presented to show the comparative calcifying powers of similar samples of green, artificially dried, and sun-cured pasture herbage. These materials were incorporated in the rations at the rate of 3, 6, and 9 percent of the total dry matter.

Pasture herbage when fed in a green, artificially dried, or sun-cured condition, constituting 3 percent of the dry matter in the ration, caused a significantly greater degree of calcification in rats than did the basal diet.

Increasing the amount of the different types of herbage fed failed to produce corresponding increases in the degree of calcium deposition.

The process of dehydration by subjecting the material to high temperatures for a short period, did not destroy the potency of the calcifying property of the herbage. Either the green or the artificially dried grass was as efficient in producing calcification as was similar herbage cured by exposure to 15 hours of sunlight.

A correlation coefficient of 0.880 ± 0.039 was found to exist between the percentage of ash in the green femur bone and that of the dry extracted bone. The correlation coefficient between the percentage of ash in the fresh femur bone and the ratio of the ash to the organic residue in the femur bone was 0.743 ± 0.025.

The result of this investigation furnished further evidence of the high nutritive value of pasture herbage.

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INJURY FROM CALCIUM ARSENATE-HYDRATED LIME SPRAY ON SNAP BEANS RETARDED IN GROWTH BY UNFAVORABLE SOIL CONDITIONS[1]

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INTRODUCTION

Observations made on commercial acreages of beans following treatments with arsenicals occasionally indicated arsenical injury in scattered sections of a field without apparent injury to the field as a whole. It appeared that a retardation in the growth of the plants associated with an unfavorable soil reaction might account for the apparent variation in susceptibility to injury. It was with this in mind that the investigation reported herein was conducted.

REVIEW OF LITERATURE

Although there is frequent mention in the literature of injury to foliage by calcium arsenate, no records have been found of previous investigations on the relation of the hydrogen-ion concentration of the soil and plant growth to the susceptibility of plants to injury from this material. Zimmerley[4] found that the range of hydrogen-ion concentration for the optimum growth of snap beans was from pH 5.3 to pH 6.0; and that the beans were chlorotic, made poor growth, and gave the lowest yields when the naturally acid soils of the Norfolk area were brought to neutral or were made slightly alkaline by the application of hydrated lime.

PROCEDURE

The experiment was conducted on the Bountiful variety of snap beans (*Phaseolus vulgaris* L.) growing in the acidity test plots of the Virginia Truck Experiment Station, a diagram of which is shown in figure 1. Treatments were applied to plots having the following approximate pH values: 7.6, 7.0, 6.5, 6.0, 5.7, 5.2, and 4.8.[5] These plots had been maintained at approximately the foregoing pH values since 1926. The plots at pH 6.5, 6.0, 5.7, 5.2, and 4.8 contained 2 check and 2 treated rows and were duplicated in the field. Those at pH 7.6 and 7.0 contained 1 check and 1 treated row and were replicated four times. Thus there were 4 treated and 4 check rows for each pH value. Grass ridges separated the pH sections, and alleys separated the plots of each section (fig. 1).

[1] Received for publication Nov. 17, 1933; issued May 1934.

[2] The writer wishes to express his appreciation to members of the staff of the Virginia Truck Experiment Station, and to Neale F. Howard, of the Bureau of Entomology, for suggestions and criticisms in the preparation of the manuscript. During the summer of 1931 the writer was assisted in the experimental work by J. R. Weedon, field assistant.

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[4] ZIMMERLEY, H. H. THE EFFECTS OF HEAVY APPLICATIONS OF PHOSPHORUS ON THE INTERRELATION OF SOIL REACTION, GROWTH, AND PARTIAL CHEMICAL COMPOSITION OF LETTUCE, BEETS, CARROTS, AND SNAP BEANS. Va. Truck Expt. Sta. Bull. 73: [861]–928, illus. 1930.

[5] The quinhydrone electrode was used in making the pH determinations. These determinations were made by workers of the horticultural department of the Virginia Truck Experiment Station.

A spray consisting of 1 pound of calcium arsenate and 2 pounds of hydrated lime in 50 gallons of water was selected for the treatments because in previous experimental work this spray had frequently caused arsenical injury to beans in this locality. Treatments were applied on September 1, 8, 15, and 21. Each treatment was applied between 1 p.m. and 5 p.m. At the time of the first treatment the beans had just begun to send out their first trifoliate leaves. The fourth treatment was applied when the plants were blooming and a few pods had formed.

A 2½-gallon compressed-air sprayer was used in applying all treatments, and the sprayer was pumped with the same number of strokes on each plot in order to maintain a uniform pressure. The insecticide was applied at the rate of 100 gallons per acre, and the

PLOT 52	PLOT 51	PLOT 50	PLOT 49	PLOT 48	PLOT 47	PLOT 46	PLOT 45	PLOT 44	PLOT 43
TREATED 2 ROWS	CHECK 2 ROWS	TREATED 1 ROW	CHECK 1 ROW	TREATED 1 ROW	CHECK 1 ROW	TREATED 2 ROWS	CHECK 2 ROWS	TREATED 2 ROWS	CHECK 2 ROWS
pH 5.2		pH 7.6		pH 7.0		pH 6.0		pH 6.5	
PLOT 38	PLOT 37	PLOT 36	PLOT 35	PLOT 34	PLOT 33	PLOT 32	PLOT 31	PLOT 30	PLOT 29
TREATED 1 ROW	CHECK 1 ROW	TREATED 1 ROW	CHECK 1 ROW	TREATED 2 ROWS	CHECK 2 ROWS	TREATED 2 ROWS	CHECK 2 ROWS	TREATED 2 ROWS	CHECK 2 ROWS
pH 7.0		pH 7.6		pH 5.2		pH 4.8		pH 5.7	
PLOT 24	PLOT 23	PLOT 22	PLOT 21	PLOT 20	PLOT 19	PLOT 18	PLOT 17	PLOT 16	PLOT 15
TREATED 2 ROWS	CHECK 2 ROWS	TREATED 1 ROW	CHECK 1 ROW	TREATED 1 ROW	CHECK 1 ROW	TREATED 2 ROWS	CHECK 2 ROWS	TREATED 2 ROWS	CHECK 2 ROWS
pH 5.2		pH 7.6		pH 7.0		pH 6.0		pH 6.5	
PLOT 10	PLOT 9	PLOT 8	PLOT 7	PLOT 6	PLOT 5	PLOT 4	PLOT 3	PLOT 2	PLOT 1
TREATED 1 ROW	CHECK 1 ROW	TREATED 1 ROW	CHECK 1 ROW	TREATED 2 ROWS	CHECK 2 ROWS	TREATED 2 ROWS	CHECK 2 ROWS	TREATED 2 ROWS	CHECK 2 ROWS
pH 7.0		pH 7.6		pH 5.2		pH 4.8		pH 5.7	

FIGURE 1.—Diagram of the experimental plots and arrangement of the pH sections.

beans were treated from each side of the row to insure thorough coverage of all parts of the plants.

Only a few adults of the Mexican bean beetle appeared in the plots. Injury by this insect was not a factor in the results.

EXPERIMENTAL RESULTS

The results of the experiment are presented in table 1. This table shows the yields of two pickings of beans on the check and treated plots of each pH value. Since the plants in each plot were counted, the percentage increase or decrease, check-plot basis, was calculated from the mean yields in grams per plant. On this basis the treatment resulted in serious reductions in yield on the plots with pH values of 7.6 and 7.0, slight reductions on plots at 6.0 and 5.2, and slight increases on plots at 6.5, 5.7, and 4.8.

TABLE 1.—*Comparative yields of snap beans, treated with an arsenical spray and untreated, grown on plots having different hydrogen-ion concentrations*

pH of soil	Plot no.		Plants per plot		Average yield per plant		Increase (+) or decrease (−) in average yield of treated plants		Estimate of injury of—	
	Check	Treated	Check	Treated	Check	Treated			Check[a]	Treated
			Number	*Number*	*Grams*	*Grams*	*Grams*	*Percent*	*Percent*	*Percent*
7.6	7		145		16.6				20	
		8		130		6.1	−10.5			[b] 60
	21		140		13.0				20	
		22		156		3.6	−9.4			[b] 70
	35		139		12.0				25	
		36		124		1.8	−10.2			[b] 80
	49		102		16.9				15	
		50		129		4.8	−12.1			[b] 60
Average					14.6	4.1	−10.5	−72		
7.0	9		169		16.1				15	
		10		201		11.0	−5.1			[b] 50
	19		177		8.5				20	
		20		124		4.6	−3.9			[b] 70
	37		149		17.1				15	
		38		163		13.7	−3.4			[b] 55
	47		157		11.9				15	
		48		145		7.6	−4.3			[b] 65
Average					13.4	9.2	−4.2	−31		
6.5	15		378		25.6					
		16		358		27.2	+1.6			[c] 10
	43		252		26.4					
		44		319		27.0	+.6			[c] 10
Average					26.0	27.1	+1.1	+4		
6.0	17		397		31.8					
		18		383		27.7	−4.1			[c] 10
	45		336		30.5					
		46		375		26.9	−3.6			[c] 10
Average					31.1	27.3	−3.8	−12		
5.7	1		385		28.0					
		2		368		28.1	+.1			[c] 10
	29		318		21.03					
		30		335		21.07	+.04			[c] 10
Average					24.51	24.58	+.07	+.3		
5.2	5		326		33.5					
		6		318		29.7	−3.8			[c] 15
	33		275		28.4					
		34		296		29.3	+.9			[c] 10
Average					30.9	29.5	−1.4	−5		
4.8	3		418		22					
		4		441		20.7	−1.3			[c] 5–10
	31		375		14.3					
		32		375		20.3	+6.0			[c] 5
Average					18.2	20.5	+2.3	+13		

[a] Estimate of chlorosis on plants.
[b] Estimate of arsenical injury and chlorosis
[c] Estimate of arsenical injury alone.

Since the check and treated plots were paired, a biometrical analysis of the data was made by Student's method of comparing two results on a probable-error basis.[6] Odds greater than 30 to 1 that the reductions were not due to chance alone were obtained on the pH 7.6, 7.0, and 6.0 plots. In the case of the first two plots, on which the reductions in yield were 72 and 31 percent, respectively, the odds were very great, approximately 5,000 to 1 and 1,300 to 1, respectively. In the

[6] HAYES, H. K., and GARBER, R. I. BREEDING CROP PLANTS. Pp. 86–89. New York. 1927.

case of the pH 6.0 plot, on which the reduction was 12 percent, the odds of a significant reduction in the yield of the treated plots under the check were only 46 to 1.

The comparative yields in grams per plant on the check and treated plots are shown in figure 2. The maximum check-plot yield was obtained on the pH 6.0 plots, and the optimum range was between pH 6.5 and 5.2.

At the conclusion of the experiment arsenical injury was slight on the pH 6.5, 6.0, 5.7, 5.2, and 4.8 plots, but was estimated at 60 to 80 percent on the pH 7.6 plots and from 50 to 70 percent on the pH 7.0 plots; thus injury was considerably more severe on the plants grown

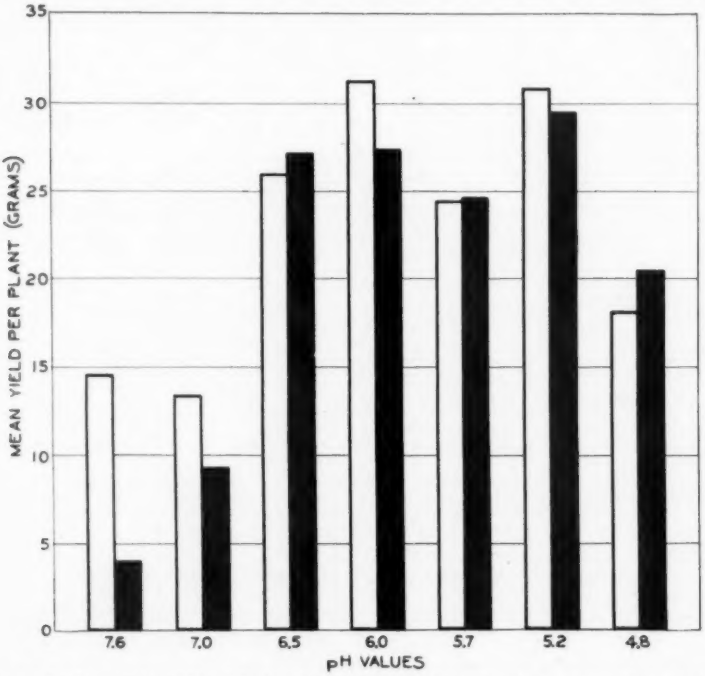

FIGURE 2.—Comparative yields of check and treated plots having different pH values. The unshaded columns represent check plots; the shaded, treated plots.

in soil at pH 7.6 and pH 7.0 than on plants grown in soil at any of the other reactions (table 1). The treated plots with pH values of 7.6 and 7.0 showed severe leaf burn, leaf shedding, and stunting.

The arsenical injury to beans on the pH 6.5 to 5.2 plots, which produced the heaviest yields (fig. 2), was not nearly so severe as has frequently been observed on beans grown under the best conditions in this locality. This is probably due to the fact that the relative humidity was comparatively low during the period of the treatments. Previous experience has shown that high humidity increases plant injury from calcium arsenate.

Serious reductions in yield occurred only on the plants grown at pH 7.6 and pH 7.0. On these plots the plants were very chlorotic on both check and treated plots, and the check yields were considerably lower than at any other pH value.

The check yield (fig. 2) from plots with a reaction of pH 6.0 was more than twice as great as the check yields from plots with a reaction of pH 7.6 and 7.0.

The results indicate that snap beans grown under the conditions of this experiment in soils of pH 7.6 and 7.0 are considerably more susceptible to injury from calcium arsenate-hydrated lime spray than are plants grown in soils of pH 6.5 to pH 4.8.

SUMMARY

Four treatments of calcium arsenate-hydrated lime spray (1:2:50) were applied to snap beans grown on soils having approximately the following pH values: 7.6, 7.0, 6.5, 6.0, 5.7, 5.2, and 4.8.

Yields were recorded, and the effect of spraying on yield, check-plot basis, was calculated from the mean yields in grams per plant. On this basis treatment was found to result in serious reductions in per plant yields on the plots with pH values of 7.6 and 7.0, slight reductions on the plots at pH 6.0 and 5.2, and slight increases on the plots at pH 6.5, 5.7, and 4.8.

A biometrical analysis of the data was made by Student's method. Odds greater than 30 to 1 that the reductions were not due to chance alone were obtained on the pH 7.6, 7.0, and 6.0 plots. In the case of the first two plots, on which reductions in yield were 72 and 31 percent, respectively, the odds were very great, approximately 5,000 to 1 and 1,300 to 1, respectively. In the case of the pH 6.0 plots, on which the reduction was 12 percent, the odds of a significant reduction in the yield of the treated plots under the check were only 46 to 1. Arsenical injury was considerably more severe on the plants grown in soil at pH 7.6 and pH 7.0 than on plants grown in soil at any of the other reactions. Severe chlorosis was apparent on check and treated plots, and check yields were considerably lower than at any other pH value.

The maximum check-plot yield was obtained on the pH 6.0 plots; the optimum range was between pH 6.5 and pH 5.2.

The results of the experiment indicate that snap beans retarded in growth by unfavorable soil conditions are decidedly more susceptible to arsenical injury from calcium arsenate-hydrated lime spray than are plants grown under optimum conditions.

THE EFFECT OF HYPERTONIC SUGAR SOLUTIONS ON THE THERMAL RESISTANCE OF BACTERIA [1]

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INTRODUCTION

In connection with the various studies on the bacteriology of ice cream which have been in progress at the Kansas Agricultural Experiment Station for several years, it has been observed occasionally that micro-organisms exhibit an increased thermal resistance when heated in ice-cream mix. Preliminary experiments showed quite definitely that certain strains of bacteria were capable of withstanding more severe heat treatment when suspended in solutions of high osmotic pressure.

The temperature and time of exposure most commonly employed in the pasteurization of ice-cream mix have been adopted from the market-milk industry without question as to the universality of their application. If thermal resistance is affected by the chemical and physical forces of the environment it is entirely logical to expect a variation in the survival of cells heated in different menstrua. The small margin of safety in the present requirements for pasteurization emphasizes the importance of evaluating any increase in thermal resistance of the microflora which may be contributed by the ingredients of ice cream.

REVIEW OF LITERATURE

Until comparatively recently the protective action of the ingredients of ice-cream mix for micro-organisms has not been recognized. Beavens (*4*) [2] found that 4- to 20-percent lactose increased the thermal resistance of *Escherichia coli*. The probability that the ingredients of ice cream might afford micro-organisms some protection against heat was recognized by the Committee on Dairy Products and Eggs (*15*). The results of Oldenbusch, Frobisher, and Shrader (*8*) gave only slight evidence of increased survival of various pathogenes when heated in ice-cream mix or in cream containing 50 percent fat. Fabian and Coulter (*6*) observed higher thermal death points for cultures of *E. coli* and *Aerobacter aerogenes* when heated in ice-cream mix than when heated in skim milk. Except for sucrose, these authors were unable to show any marked protective effect when the ingredients of ice cream were studied separately.

Anzulovic (*1*) reported that sugar, gelatin, serum solids, and fat showed some protective action for bacteria. Weiss (*17*) found that *Bacillus botulinus* was more resistant to heat in foods containing heavy sirups. Rahn (*9*) stated that—

[1] Received for publication Oct. 17, 1933; issued May 1934. A résumé of a dissertation submitted to the graduate faculty of Iowa State College in partial fulfillment of the requirements for the degree of doctor of philosophy. The work was made possible through the cooperation of the Department of Dairy Industry, Iowa State College of Agriculture and Mechanic Arts, and the Department of Bacteriology, Kansas State College of Agriculture and Applied Science. Published as doctoral dissertation no. 244, Iowa State College, and contribution no. 154 of the Department of Bacteriology, Kansas State College.

[2] Reference is made by number (italic) to Literature Cited, p. 467.

* * * sugar not only retards growth of yeasts and other micro-organisms, but also retards the action of heat upon micro-organisms; it will take more heat to kill a bacterium or yeast in a sweetened fruit juice than in the same juice without sugar.

Robertson (*11, 12, 13, 14*) heated *Streptococcus thermophilus*, *Sarcina lutea*, *Escherichia coli*, and *Micrococcus aureus* in increasing percentages of sugar and found that as the concentration of sugar was increased the number of surviving bacteria also increased.

Nechkovitch (*7*) showed that glucose tended to prevent the coagulation of cell colloids and aided in maintaining a normal condition of stability of the cells of organized tissue. Wallace and Tanner (*16*) suspended several species of molds in 10-, 25-, and 50-percent sugar, distilled water, sirups from fruit juices, and in salt water. Protective action was afforded by sugar for some molds and by salt water for others.

Rahn (*10, p. 330*) reported increased thermal resistance of yeasts and bacteria when heated in broth containing 50 percent of sucrose. This author suggested that although death may be due in part to dehydration, the cause of death is not the same as with dry bacteria. Cook[3] explained his observations of increased resistance of yeasts in hyper tonic glucose and sucrose solutions on the basis of dehydration of the cells. He stated, however, that there was probably some factor other than osmotic pressure involved, since the killing times were not proportional to the osmotic pressure of the solutions employed.

Beilinsson (*5*) observed that the addition of sufficient sucrose or glycerol to serum albumin or to egg white rendered these proteins stable to temperatures far above the usual coagulation points. Bancroft and Rutzler (*3*) and Bancroft and Richter (*2*) confirmed these observations and suggested an explanation based upon the peptizing action of sugar on albumin. According to these authors, if the colloidal suspension is reversibly coagulated, the cell may have lost temporarily any or all of its vital manifestations, but will recover from dormancy when placed in a favorable environment. As agglomeration of the cell contents increases, the cell loses more and more of its functions, the coagulation becomes progressively less reversible and finally completely irreversible.

METHODS

In all the plating procedures standard beef-extract agar was employed as the basic medium. In those instances in which comparisons were made between the counts on plain and carbohydrate media, a large batch of the plain agar was divided into equal parts and 1 percent of the desired carbohydrate added to each portion. The reaction of all media was adjusted to pH 7.0 before filtration.

The distilled water used in making dilution blanks occasionally was tested after sterilization for its reaction and found to be pH 6.0 ± 0.2.

All plates were incubated 48 hours at 37° C. In several of the experiments where delayed germination was suspected, counts were made again after an additional 3 days' incubation at room temperature.

In several experiments reference is made to the use of a 100-percent sugar solution. This refers to a weight-volume ratio, and the solution was prepared in the following manner: Approximately 250 cc of water was added to 1 kg of the desired sugar, and this was boiled for a few

[3] COOK, W. B. STUDIES ON THE STERILIZATION OF SOLUTIONS OF GLUCOSE AND SUCROSE. (Thesis, Ph. D., Iowa State College.) 1931.

minutes until a clear solution was obtained. The resulting sirup (approximately 900 cc) was diluted to 1,000 cc total volume. Such a solution therefore contained 1,000 g of sugar in a total volume of 1,000 cc, and each cubic centimeter represented 1 g of sugar. All the other sugar solutions employed, the concentrations of which are expressed in percentages, were prepared by suitable dilution of this 100-percent sugar solution. The sugar solutions, the concentrations of which are expressed in molality, were prepared by dissolving the indicated number of gram-molecular weights in 1,000 cc of water.

In order to reduce the factor of heat penetration to a practical minimum, small samples were used in all heating trials. In some cases, 1.5 to 2.0 cc samples were placed in small hermetically sealed tubes and completely submerged in a water or an oil bath for the desired heating period. In other cases, thin-walled, small-bore (4 mm) test tubes were submerged in the oil bath to within 1 inch of the top of the tube. For many of the experiments special tubes were prepared by blowing a bulb about 1.5 inches in diameter on the end of a soft glass test tube. The small sample in the relatively large bulb of very thin glass acquired the temperature of the water bath very quickly. Obviously, heat penetration would be delayed in samples containing high percentages of sugar. However, the importance of this factor was shown to be reduced to a negligible minimum when small samples (2 cc or less) were employed. Graphs illustrating the rates of heat penetration under the conditions of these experiments show practically superimposed lines.

Although the results of the preliminary experiments did not indicate that it was necessary, all tubes containing more than a 0.1 cc sample were agitated by uniform shaking during the heating period.

RESULTS

HYPERTONIC SOLUTIONS IN ICE CREAM MIX

A sample of ice cream mix which contained no sucrose was incubated at room temperature until the bacterial count reached several million per cubic centimeter. This was divided into four parts and equal volumes of sterile sucrose solutions were added to give 15-, 25-, and 50-percent concentrations of sugar. A similar volume of water was added to one portion, which was designated as 0-percent sucrose. Plates were made before and after heating small portions of the four samples simultaneously to 54.5° C. for 9 minutes. In order to facilitate comparison, the number of viable organisms after heating has been calculated on the basis of survival per million.

The data in table 1 were compiled from the results of four such trials, and illustrate the general trend of many similar experiments. It will be observed in experiment 1 that the ice cream mix had a plain agar plate count of 14,000,000 per cubic centimeter before heating. After heating, the survival per million was 450 in the sample containing no sucrose, and 35,000, 44,000, and 35,000 in the samples containing 15-, 25-, and 50-percent concentrations of sucrose, respectively. Since all four samples were identical except for the sugar, the results suggest that the presence of sugar in ice cream renders the cells more resistant to heat.

TABLE 1.—*The effect of plain and carbohydrate agars on the survival of micro-organisms in ice cream mix containing various amounts of sugar after heating at 54.5° C. for 9 minutes*

Experiment no.	Agar	Count per cubic centimeter before heating	Survival per million after heating in ice cream mix containing—			
			0 percent sucrose	15 percent sucrose	25 percent sucrose	50 percent sucrose
		Millions	*Number*	*Number*	*Number*	*Number*
1	Plain	14	450	35,000	44,000	35,000
2	Plain	170	310	1,500	2,800	25,000
	Dextrose	180	19,000	14,000	14,000	61,000
	Sucrose	130	24,000	18,000	22,000	77,000
	Lactose	170	19,000	18,000	22,000	59,000
3	Plain	110	220	5,500	5,900	44,000
	Sucrose	110	5,500	8,000	8,000	39,000
4	Plain	66	260	7,900	7,900	18,000
	Sucrose	60	17,000	18,000	17,000	18,000

In the other experiments reported in table 1 the same routine was followed except that various carbohydrate agars were employed in pouring parallel plates from dilution blanks. In experiment 2, for example, the survival per million in the sample containing no sucrose was 310 when plated on plain agar, and from 19,000 to 24,000 on carbohydrate agars. This at once suggests that some of the cells, injured but not irreparably destroyed by the heat, were capable of recovery when the medium was fortified with a carbohydrate. In the samples containing 15- and 25-percent sucrose the plain agar counts per million were 1,500 and 2,800, respectively, which indicates that the presence of sugar in the sample diminished the degree of injury to the cells and enabled more of them to recover even in plain agar. Transfers from the same dilution blank to carbohydrate media gave survival per million values ranging from 14,000 to 22,000, again emphasizing the importance of the medium in the recovery of injured cells.

The protective action of sugar is most effectively illustrated in the samples containing 50 percent of sucrose. The thermal resistance of the cells was greatly increased in the presence of such excessive amounts of sucrose.

A consideration of the results of experiments 3 and 4 leads to the same general observations, viz, in ice-cream mix without sugar injury of the cells by heat is greater than when sugar is present; the number of organisms capable of surviving increases with increasing percentages of sugar in the mix (this is especially noticeable when 50 percent of sugar is employed); and more of the injured cells survive in carbohydrate media.

If high osmotic pressures result in an increased thermal resistance for cells, it is conceivable that micro-organisms whose normal resistance is at the threshold of the thermal exposure of pasteurization may survive in ice cream and not in milk.

HYPERTONIC SOLUTIONS IN MILK

Sterile milk was heavily inoculated with a pure culture of *Escherichia coli* 57 and then diluted (1) with an equal volume of water, and (2) with an equal volume of 100-percent sucrose, thereby giving an ultimate concentration of 50-percent sugar. These two samples were

plated before and after heating (54.5° C., 9 minutes) on plain agar and on 1-percent dextrose, sucrose, and lactose agars.

The results in table 2 show that approximately 5 to 10 times as many organisms survived in the milk to which sucrose was added. In this experiment the use of carbohydrate agar did not increase the number of survivors—an observation that tends to discourage the conclusion that the injured cells are necessarily rendered more saccharophilic.

TABLE 2.—*Effect of various media on the recovery of Escherichia coli 57 heated at 54.5° C. for 9 minutes in suspensions of milk and milk containing 0- and 50-percent sucrose*

Medium	Count per cubic centimeter before heating		Survival per million after heating at 54.5° C. for 9 minutes	
	0-percent sucrose	50-percent sucrose	0-percent sucrose	50-percent sucrose
Plain	10,000,000	10,000,000	48,000	250,000
Dextrose	14,000,000	11,000,000	20,000	230,000
Sucrose	10,000,000	11,000,000	18,000	245,000
Lactose	12,000,000	15,000,000	30,000	150,000

HYPERTONIC SOLUTIONS IN BROTH

PROTECTIVE ACTION OF VARIOUS SUGARS FOR ESCHERICHIA COLI HELD FOR VARIOUS LENGTHS OF TIME

Uniform suspensions of *Escherichia coli* 25 were prepared in plain broth and in broths containing 50 percent, respectively, of maltose, sucrose, and dextrose. Because of the low solubility of lactose, suspensions of cells in saturated lactose broth were prepared separately. These suspensions were plated as quickly as possible on plain agar before and after heating to 54° C. for 9 minutes. After the original suspensions had stood at room temperature for 2 hours, plates were again made before and after heating to 54° for 9 minutes.

Table 3 shows that saturated lactose failed to protect *Escherichia coli* 25, and that 50-percent maltose broth afforded only very slight protection. The survival per million values in 50-percent sucrose and in 50-percent dextrose broths were increased ninefold and sevenfold, respectively, after the cultures had aged 2 hours.

TABLE 3.—*Thermal resistance of Escherichia coli 25 after 2 hours' contact with plain broth, saturated lactose broth, and 50-percent maltose, sucrose, and dextrose broths*

Cell suspension	Count per cubic centimeter after—				Survival per million after—	
	0 hour contact		2 hours contact			
	Before heating	After heating at 54° C. for 9 minutes	Before heating	After heating at 54° C. for 9 minutes	0 hour contact	2 hours contact
Plain broth	500,000	50	550,000	730	100	1,330
Saturated lactose broth	500,000	30	520,000	0	60	0
50-percent maltose broth	500,000	1,100	130,000	380	2,200	2,900
50-percent sucrose broth	340,000	9,800	220,000	58,000	29,000	264,000
50-percent dextrose broth	600,000	18,000	60,000	13,000	30,000	217,000

EFFECT OF PROLONGED EXPOSURE OF CELLS TO HYPERTONIC SUCROSE SOLUTION

Suspensions of *Escherichia coli* 52 in plain and 50-percent sucrose broths were held at 30° C. Samples were removed from the sucrose suspension after each 15-minute interval for 2 hours and then at less frequent intervals for 7 hours. Each sample removed was plated on plain agar before and after heating at 54.5° for 5 minutes.

A study of the results presented in table 4 shows a marked increase in the thermal resistance during the first 2 hours. It is significant to note that although the counts on the sucrose broth before heating were fairly constant, the actual number of cells capable of surviving the heat treatment increased. Evidently the physical changes responsible for greater heat stability of the protoplasm affect an increasing number of cells with time. In this instance the maximum number of cells capable of withstanding the heat treatment occurred after 2 hours' exposure, whereas continued contact with the sugar resulted in a decrease in the thermal resistance.

TABLE 4.—*The thermal resistance of Escherichia coli 52 after prolonged incubation at 30° C. in plain and in 50-percent sucrose broth before heating at 54.5° C. for 5 minutes*

Period of contact before heating (minutes)	Count per cubic centimeters in—				Survival per million in—	
	Plain broth		50-percent sucrose broth			
	Before heating	After heating at 54.5° C. for 5 minutes	Before heating	After heating at 54.5° C. for 5 minutes	Plain broth	50-percent sucrose broth
0	1,500,000	10	1,700,000	42,000	7	25,000
15			1,700,000	170,000		100,000
30			1,800,000	450,000		250,000
45			2,200,000	570,000		259,000
60	2,600,000	0	2,300,000	750,000	0	326,000
75			1,700,000	870,000		512,000
90			1,800,000	960,000		533,000
105			1,700,000	690,000		406,000
120	4,100,000	0	1,400,000	1,100,000	0	786,000
180	38,000,000	0	2,900,000	550,000	0	190,000
240	150,000,000	0	1,200,000	350,000	0	292,000
300	220,000,000	10	1,100,000	210,000	0	191,000
420	330,000,000	0	930,000	57,000	0	61,000

The values for the survival per million in the 50-percent sucrose broth have been plotted against time in figure 1. The graph clearly shows the tendency for the cells to become progressively more resistant to heat when exposed to hypertonic sucrose solutions for 2 hours and the tendency to lose this faculty on continued exposure.

These results suggest that high concentrations of sucrose may induce a physical change which no doubt is regulated by the permeability of the individual cell. This process, although ultimately leading to the death of the cell, at first increases the stability of the protoplasm to heat. Continued exposure, however, advances the degree of coagulation to a point beyond which plain agar can no longer induce peptization. The ultimate decline in the number of cells capable of surviving the heat treatment apparently is the result of prolonged desiccation beyond the point of optimum stability.

HYPERTONIC SOLUTIONS IN WATER

EFFECT OF VARIATIONS ON OSMOTIC PRESSURE

Escherichia coli 52 was suspended directly in hypertonic solutions of dextrose and sucrose of various molalities. One tenth cubic centimeter of an 18-hour plain-broth culture was placed in 5-cc quantities of the various sugar solutions; after thorough agitation these were allowed to stand at room temperature for 5 minutes. Two cubic centimeter portions of each suspension were then heated to 55° C. for 5 minutes. Plate counts were made on plain and dextrose agars before and after heating. The data, calculated to a basis of survival per million, are presented in table 5. Owing to the fact that each solution was inoculated separately, there was some unavoidable variation in the initial number of organisms in the solutions

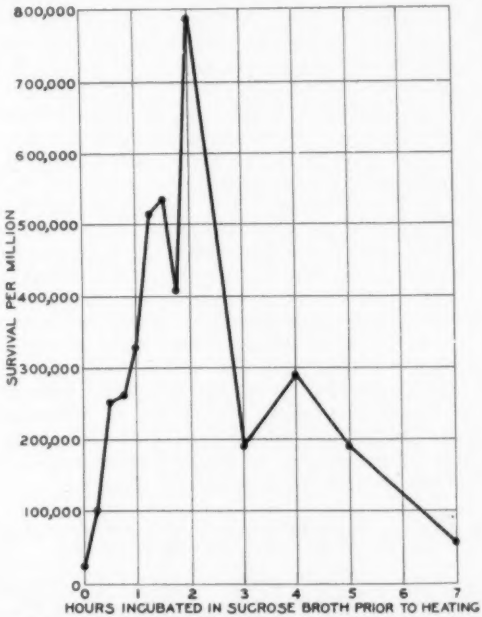

FIGURE 1.—Thermal resistance of *Escherichia coli* 52 after prolonged incubation at 30° C. in 50-precent sucrose broth before heating at 54.5° C. for 5 minutes.

TABLE 5.—*Survival per million of Escherichia coli 52 when heated in equimolal dextrose and sucrose solutions*

Molality of sugar solutions	Survival per million of *E. coli* when heated in—				Molality of sugar solutions	Survival per million of *E. coli* when heated in—			
	Dextrose solutions		Sucrose solutions			Dextrose solutions		Sucrose solutions	
	Plain agar	Dextrose agar	Plain agar	Dextrose agar		Plain agar	Dextrose agar	Plain agar	Dextrose agar
0.25	0	0	180	0	1.5	4,700	12,000	170,000	210,000
0.50	0	450	0	180	2.0	15,000	15,000	360,000	330,000
0.75	0	600	270	21,000	3.0	47,000	31,000	270,000	320,000
1.0	90	360	11,000	100,000	4.0	150,000	100,000	150,000	190,000

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Since the osmotic pressures of the equimolal solutions were identical the differences observed in the protective action cannot be accounted for on this basis. It is possible, however, that there were differences in the effective osmotic pressure at the cell surfaces, since this would depend upon the permeability of the individual cell membrane. These results are in harmony with those of Cook[4] to which reference has already been made.

It is evident from table 5 that very little protective action is afforded by either sugar in concentrations below 1 molal. The values in table 5 show quite forcibly that there is an increased protective action with the increasing osmotic pressure beyond 1-molal concentrations.

Effect of Adding Sugar After Heating

In order to determine whether the increased survival of cells heated in hypertonic sugar solutions was attributable to the sugar carried over from the sample into the medium, the following experiment was performed. A 24-hour plain broth culture of *Escherichia coli* 52 was diluted with an equal volume of water in one tube, and with an equal volume of 100-percent sucrose solution in another. These cultures were heated to 54.5° C. for 5 minutes. After heating, the water suspension was divided into two equal parts; an equal volume of 100-percent sucrose solution was added to one part, and the other was similarly diluted with water. This provided (1) cells which had been heated in water and then the sugar added (50-percent concentration) after heating, and (2) cells heated in water and subsequently further diluted with water. In a similar manner the original suspension of cells which had been heated in 50-percent sucrose solution was divided into two parts, one of which was diluted with an equal volume of 50-percent sucrose solution and the other with an equal volume of water. If cells suspended in sucrose surround themselves with a layer of sugar which ultimately serves as an intimate source of energy in the agar plate, one would expect the suspension to which sugar was added after heating to exhibit approximately the same survival as the cells heated in the presence of sugar.

Table 6.—*Effect of adding 50-percent sucrose to cell suspensions of Escherichia coli 52 after heating at 54.5° C. for 5 minutes*

[Controls suspended in water]

Treatment of cells	Agar	Count per cubic centimeter		
		Before heating	After heating at 54.5° C. for 5 minutes [a]	Survival per million
Heated in 50-percent sucrose, then equal volume of 50-percent sucrose added.	Plain	78,000,000	2,600,000	67,000
	Dextrose	80,000,000	6,700,000	168,000
Heated in water, then equal volume of 50-percent sucrose added.	Plain	190,000,000	300,000	3,200
	Dextrose	230,000,000	260,000	2,300
Heated in water, then equal volume of water added.	Plain	190,000,000	2,100,000	22,000
	Dextrose	230,000,000	1,100,000	10,000

[a] Since the samples were diluted with equal volumes of sucrose solution or water after heating, the values in this column should be doubled. The values for survival per million have been adjusted accordingly.

[4] Cook, W. B. See footnote 3.

The results in table 6 show that the addition of sugar after the cells had been heated in water failed to induce recovery from injury; in fact, the values for survival per million were actually smaller than those observed in the water suspension. The damage to the cells heated in water apparently cannot be counteracted by the subsequent addition of sugar. If the sugar is present during the heating a relatively large number of the cells are protected from irreparable injury.

Effect of Hypertonic Sugar Solutions on Thermal Resistance of Various Bacteria

Some of the results already presented suggest that the permeability of the individual cell membrane may play an important part in the phenomenon of protective action. The results of several experiments not presented in this paper have suggested further that the protective action of dextrose and sucrose solutions was best demonstrated with organisms which were sensitive to heating in water. Considering these individual variations one would not expect, therefore, that all bacteria would be protected by hypertonic solutions.

The protective action of dextrose and maltose was tested on pure cultures of several different organisms. One cubic centimer of an 18-hour plain broth culture of the test organism was inoculated into each of three flasks containing 40 cc of water, 2-molal dextrose, and 2-molal sucrose, respectively. After thorough agitation samples were removed for plating and heating. The time of heating was 5 minutes in all cases, but the temperature employed varied with the different organisms.

Table 7.—*Effect of hypertonic solutions on the thermal resistance of cultures of several different organisms*

Culture	Count per cubic centimeter before heating in—			Temperature of exposure for 5 minutes	Count per cubic centimeter after heating in—		
	Distilled water	2-molal dextrose	2-molal sucrose		Distilled water	2-molal dextrose	2-molal sucrose
				° C.			
Serratia marcescens	28,000,000	23,000,000	17,000,000	55	100	540,000	160,000
Staphylococcus albus	40,000	16,000	60,000	55	0	0	0
Pseudomonas fluorescens	30,000	1,000	12,000	55	10	60	9,000
Aerobacter aerogenes	12,000,000	14,000,000	10,000,000	60	2,800	35,000	75,000
Staphylococcus aureus	13,000,000	37,000	7,900,000	60	120	190	2,600
Bacillus subtilis	130,000	130,000	140,000	90	20	130	500
Salmonella pullorum	1,000,000	1,500	1,000	55	190,000	1,100	1,200

The data in table 7 show striking variations in the effect of water, 2-molal dextrose, and 2-molal sucrose on thermal resistance of cells. First, it will be noted that there is little or no evidence that either dextrose or sucrose afforded the strains of *Staphylococcus albus* or *Salmonella pullorum* any increased resistance to heat. However, it should be noted that the counts before heating in the dextrose and sucrose suspensions of *S. pullorum* are practically the same as those obtained after heating. When compared with the counts on the aqueous suspensions before heating it is evident that very large percentages of the organisms died almost instantly when introduced into the sugar solutions, but those which did survive were able to withstand the heat treatment.

There is only slight evidence that 2-molal sucrose protected *Bacillus subtilis*, whereas the results with *Pseudomonas fluorescens* and *Staphylococcus aureus* indicate protective action more definitely. *Serratia marcescens* and *Aerobacter aerogenes* were unmistakably protected by the presence of either dextrose or sucrose. Two-molal dextrose apparently offered more effective protection to *S. marcescens* than did an equimolal solution of sucrose; the reverse was true, however, for *A. aerogenes*. In each case in which protective action was observed the cells exhibited extreme sensitivity to heat in water suspensions.

Effect of Washing Cells After Exposure to Sugar

Five cubic centimeters of an 18-hour plain broth culture of *Escherichia coli* 52 were centrifuged and all but 1 cc of the supernatant broth removed. One cubic centimeter of 100-percent sucrose was added and the resulting 50-percent sugar suspension of cells allowed to stand at room temperature for 30 minutes. The cells were then washed seven times with 0.85-percent NaCl solution to remove the sugar. At the appropriate time two 5-cc portions of a broth culture of the same organism were centrifuged to concentrate the cells. All but 1 cc of the supernatant broth was removed from one of these tubes and 1 cc of the 100-percent sucrose added as before. The cells were allowed to remain in contact with the sugar for 30 minutes at room temperature. The other tube of centrifuged cells was used as a broth control. In this case all but 2 cc of the supernatant broth was removed and the cells resuspended in the 2 cc volume of broth. After 1-cc portions of these three suspensions had been removed for plating before heating, the tubes were placed in the water bath at 54.5° C. for 5 minutes. Plates before and after heating were made with plain and dextrose agars.

Table 8.—*Thermal resistance of Escherichia coli 52 when exposed to 50-percent sucrose 30 minutes, then washed in 0.85-percent NaCl*

Treatment of cells	Agar	Count per cubic centimeter—		Survival per million
		Before heating	After heating at 54.5° C. for 5 minutes	
Exposed to 50-percent sucrose 30 minutes, washed 7 times, and heated in 0.85-percent saline.	Plain	280,000,000	340,000	1,200
	Dextrose	320,000,000	410,000	1,300
Exposed to 50-percent sucrose 30 minutes, not washed, heated in the sucrose solution.	Plain	840,000,000	400,000,000	476,000
	Dextrose	900,000,000	400,000,000	444,000
Centrifuged from broth, resuspended, and heated in broth.	Plain	1,000,000,000	1,300,000	1,300
	Dextrose	960,000,000	4,200,000	4,400

It is quite evident from the data in table 8 that the protective action which 50-percent sugar afforded cells was readily removed by washing the cells in an 0.85-percent NaCl solution. The cells heated in saline and in broth suspensions were quite susceptible to heat, whereas the presence of sugar during heating resulted in increased resistance.

Protective Action of Hypertonic Solutions Against the Coagulation of Nonliving Protein Systems

It is obviously difficult to demonstrate changes in the physical status of the colloidal system of a minute bacterial cell except by indirect means. If the coagulation of cell colloids follows simple,

well-defined laws of colloid chemistry, then the protective action afforded by sugars should be readily demonstrable with nonliving colloids. The following experiments were designed to investigate such a possible parallelism.

Effect of Hypertonic Dextrose and Sucrose Solutions on the Coagulation of Egg Albumin

By serial dilution of 8-molal sucrose and dextrose solutions, 6-, 4-, 3-, 2-, 1-, 0.5-, 0.2-, and 0.1-molal concentrations of each sugar were prepared. From these, series of tubes were arranged each containing 1 cc of one of the foregoing concentrations of sugar. To each tube 1 cc of fresh egg albumin was added and thoroughly mixed with the sugar solution, thus reducing the sugar to one half its original concentration. Tubes containing a mixture of equal parts of water and egg albumin, and also tubes contained undiluted egg albumin were used as controls.

The series of tubes for both sugars were placed simultaneously in a water bath at the desired temperature and the time of coagulation noted. It was necessary to adopt an arbitrary standard of turbidity for coagulation in order that the readings might be rendered uniform. The albumin was arbitrarily regarded as coagulated when it was no longer possible to identify and differentiate the letters on a typewritten page held behind the tube; the diameter of the tube was 10 mm.

Table 9.—*Effect of equimolal concentrations of dextrose and sucrose on the coagulation of egg albumin*

Molality of sugar	Time required to coagulate egg albumin at—					
	60° C.		65° C.		100° C.	
	Dextrose	Sucrose	Dextrose	Sucrose	Dextrose	Sucrose
	Minutes	*Minutes*	*Minutes*	*Minutes*	*Minutes*	*Minutes*
4	(a)	(a)	(a)	(a)	0.5	0.5
3	(a)	(a)	34	52	.2	.2
2	73	(a)	8	11.5	.2	.2
1	23	31	4	4.5	.2	.2
0.5	8	10	3	3.5	.2	.2
0.2	6	7	3	3.5	.2	.2
0.1	6	7	3	3.5	.2	.2
0.05	5	5	2	2	.2	.2
Water, egg 1:1	3	3	2	2	.2	.2
Undiluted egg	4	4	2	2	.2	.2

[a] Not coagulated after 24 hours.

The data in table 9 show the time required for coagulation of egg albumin at 60°, 65°, and 100° C. when mixed with equal parts of various equimolal concentrations of dextrose and of sucrose. At 60° a material increase in the time required for coagulation was observed when the sugars were present in 1-molal or greater concentrations. When 3-molal and 4-molal solutions of either sugar were employed coagulation was prevented for at least 24 hours. It is especially significant that sucrose afforded more protection than dextrose. The albumin coagulated in 73 minutes at 60° in the presence of 2-molal dextrose, whereas an equimolal solution of sucrose prevented coagulation for 24 hours.

When the experiment was repeated at 65° and 100° C. much more rapid rates of coagulation were observed. For example, with the 3-molal solutions the albumin failed to coagulate in 24 hours at 60°, but when exposed to 65° coagulation occurred in 34 minutes in the dextrose and 52 minutes in the sucrose solutions. At 100° coagulation in all concentrations employed occurred in relatively few seconds.

Effect of Various Solutes on the Inactivation of Rennin by Heat

To a series of tubes each containing 2 cc of rennet extract was added an equal volume of one of the following substances: Conductivity water, 1-molal sodium chloride, 1-molal calcium chloride, 4-molal sucrose, and glycerol (specific gravity 1.25). The tubes, together with a control tube containing undiluted rennet, were placed in a water bath at 70° C. At various time intervals one drop of the rennet mixture from each tube was removed and added to tubes containing 10 cc of fresh, raw milk. The tubes of milk were held at room temperature and observed for the time of coagulation. Progressive inactivation of the rennin increased the time required to coagulate milk.

Table 10.—*Effect of various substances on the inactivation of rennin by heat*

[Material added to rennet, equal volume]

Period rennet mixture was exposed to 70° C. (minutes)	Coagulation time of 10 cc of milk by 1 drop of rennet mixture					
	Nothing added	Conductivity water	1-molal sodium chloride	1-molal calcium chloride	4-molal sucrose	Glycerol
	Minutes	*Minutes*	*Minutes*	*Minutes*	*Minutes*	*Minutes*
0	34	30	29	20	33	20
2.5	40	72	41	22	32	24
5.0	109	(a)	319	22	31	31
7.5	162	.....	(a)	36	27	36
10.0	314	.....	.....	39	25	100
12.5	550	.....	.....	101	61	106
15.0	(a)	.....	.....	109	70	135
20.0	.....	.....	.....	230	65	260
30.0	.....	.....	.....	594	55	(a)
45.0	.....	.....	.....	(a)	40	.....
60.0	.....	.....	.....	.....	35	.....
100.0	.....	.....	.....	.....	50	.....
120.0	.....	.....	.....	.....	50	.....
150.0	.....	.....	.....	.....	44	.....
180.0	.....	.....	.....	.....	70	.....
240.0	.....	.....	.....	.....	55	.....
300.0	.....	.....	.....	.....	60	.....

[a] Not coagulated after 24 hours.

It will be observed from the data in table 10 that 1 drop of the undiluted rennet before heating induced coagulation of 10 cc of milk in 34 minutes. After the milk had been heated for 2.5, 5.0, 7.5, 10.0, and 12.5 minutes, the coagulation times increased to 40, 109, 162, 314, and 550 minutes, respectively. After exposure to 70° C. for 15 minutes the rennin was so completely inactivated that it could not induce coagulation in 24 hours. All the milk not coagulated after 24 hours was still sweet to the taste.

The rennet extract which was diluted with water was completely inactivated after 5 minutes' exposure. This agrees with the decreased thermal resistance observed with certain bacterial cells in water suspension. Likewise, it will be observed in table 10 that 4-molal

sucrose very effectively protected rennin against destruction by heat. Even after 5 hours' exposure at 70° C. the rennin was sufficiently active to coagulate milk in 60 minutes. Glycerol and 1-molal calcium chloride also afforded definite protective action, although not to the same degree as sucrose. Sodium chloride apparently hastened the inactivation of the rennin. The data for suitable control tubes were not incorporated in the table as the coagulation times were identical with those observed for the undiluted rennet, except in the case of the milk to which calcium chloride was added. As might be expected, the addition of calcium chloride to the milk reduced the time of coagulation a few minutes in each case. The observations are so completely in accord with those made with cell suspensions that the possibility of the operation of the same set of factors is forcibly impressed.

DISCUSSION

The data in this paper lend support to the concept that the individual cell does not die suddenly after a given exposure to heat. Unfavorable environmental factors induce changes in the cells, which behave in accordance with well-established principles of colloidal chemistry. It is believed that all the salient features of the data presented can be explained on the basis of the theory of cell destruction outlined by Bancroft and Richter (*2*). This concept permits of a logical basis for elucidating protective action, increased growth in carbohydrate media, sensitivity of cells to water, and other observations made in connection with this study.

The degree of dispersion of the colloids of the normal cell presumably is at least nearly optimum for maximum stability. As the agglomeration of the colloids becomes progressively more pronounced, the cell becomes more sluggish and eventually dormant. Such a cell is narcotized and may be revived if placed under conditions conducive to peptization of the colloids to their normal degree of dispersion.

It is apparent that the more advanced the degree of coagulation, the greater will be the difficulty encountered in peptization. Similarly, one should expect decided differences in the peptizing qualities of various media. The coagulated colloids of an injured cell may be peptized by one medium and not by another. Obviously, reversibility of coagulation is a relative matter and depends upon a complementary relationship with the peptizing qualities of the medium employed.

A summary view of the data presented in this paper suggests that certain sugars retard the agglomerating action of heat on the protoplasmic colloids. As a result, cells subjected to a given heat treatment in concentrated sugar solutions are only reversibly coagulated, whereas in aqueous suspensions the protoplasm more closely approaches the irreversible stage of coagulation. In these experiments thermal exposures have been employed which were barely adequate to kill the cells in aqueous suspensions, thereby favoring a demonstration of the maximum protective action in the sugar suspensions. Experiments are now in progress with cultures whose heat resistance is just at the threshold of the thermal exposure of pasteurization. If some of the organisms in ice-cream mix are sufficiently protected by the presence of sugar to enable them to survive, the need for a readjustment of pasteurization requirements for this product would be suggested. The practical aspect of the protective action of sugar is also mani-

fested in the production of sweetened condensed milk, and the preservation of foods containing high percentages of sugar such as fruits canned in sirup, honey, molasses, etc.

The protective action of hypertonic sugar solutions against the action of heat on egg albumin and rennin further suggests that the protection is afforded by retarding the rapid coagulation of the colloidal complex. The addition of water hastened the time of coagulation or destruction of the enzyme, and the addition of hypertonic sugar solutions retarded the same process. The parallelism between the observation with nonliving proteins and those made with living cells is quite apparent. The precise mechanism by which sugar solutions retard the coagulation of nonliving proteins is one which still challenges the attention of physical chemists. The data presented in this paper suggest that the same fundamental principles involved may apply to the destruction of bacteria.

When the cell colloids have agglomerated to the extent that all efforts fail to peptize them, the cell may be regarded, with reservations, as irreversibly coagulated or dead. The impracticability of proving such a point beyond all peradventure of doubt is illustrated by the many reports in the literature of delayed germination of heated cells. Although it is quite proper to conclude that the colloids of a cell are irreversibly coagulated with respect to a given medium, obviously the conclusion should be confined to the observation, especially if minimal exposures have been employed. Any determination of minimum lethal exposure is subject to question on the basis of the uncertainty of the death of the organism.

The importance of permeability in protective action is suggested by the results of several experiments. Prolonged exposure of certain cells to hypertonic solutions results in an actual increase in the number of cells capable of withstanding a given heat treatment, although the total number of cells in the unheated samples may show a steady decline. It is believed that this is explicable on a basis of different degrees and rates of permeability of the cells in the population. The variations observed in the protective action of various sugars for different organisms also suggest that penetration of the cell wall must play an important role in the regulation of protective action.

When certain cells are heated in a series of solutions of dextrose with increasing osmotic pressures, there is an unmistakable parallel increase in the protective action. A similar series of equimolal concentrations of sucrose will show considerably greater protective action than the dextrose solutions. This suggests that although osmotic pressure is an important factor in the protective action afforded by sugars it is not the only agency involved. If the protective action is to be explained on a basis of the transfer of water, obviously osmotic pressure would play an important part. On the other hand, the effective osmotic pressure at the cell surface is regulated not only by the molecules in solution but by the relative permeability at each individual cell surface.

SUMMARY AND CONCLUSIONS

This work involves a study of the increased resistance manifested by certain micro-organisms when heated in the presence of high concentrations of sugars. The significance of this phenomenon

in the heating of ice-cream mix is demonstrated and the probable relation to the preservation of condensed milk, canned fruits, honey, molasses, etc., is suggested.

Within the limitations imposed by the experimental procedure it has been possible to demonstrate some of the fundamental points bearing on the mechanism of this protective action. The permeability of the individual cell apparently plays an important part in regulating the rate of death in water and in sugar suspensions.

The protective action increased with increased osmotic pressure in a series of concentrations of a given sugar, but equimolal solutions of different sugars did not show the same protective action. Maltose and lactose gave little or no protection to the cells studied.

Not all cells exhibit the phenomenon of increased thermal resistacne in hypertonic solutions. Such resistance is believed to be limited to those cells which are highly sensitive to water at slightly increased temperatures. Washing of cells after exposure to hypertonic solutions removes any protective action which the sugar solution affords the cells in the unwashed portion.

Parallel with the protective action for cells, hypertonic sugar solutions have been shown to delay or even prevent the coagulation of egg albumin and to retard the inactivation of rennin by heat.

The general conclusions from these data may be briefly stated as follows: The addition of sugar to ice-cream mix may increase the thermal resistance of the microflora. Hypertonic solutions of dextrose and sucrose made up in broth, milk, water, or ice-cream mix afforded certain cells definitely greater protection against heat than when heated in water suspensions. The addition of sugar after heating did not induce an increased survival of the cells employed in this study. The variation in the peptizing qualities of different media for the cells which have been only reversibly coagulated by minimal exposures suggests caution in all studies based upon the death of the cell as measured by cultural methods.

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THE INFLUENCE OF LIME ON THE REACTION OF SUBSOILS[1]

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INTRODUCTION

Much has been learned of the effect of lime on the soil, especially on the part that is constantly being stirred by the plow and other implements of cultivation. Less is known, however, of its effect on the soil that lies immediately beneath the plowed depth. Work at Rothamsted, England,[2] at Cornell,[3] Florida,[4] and other experiment stations has shown that lime is lost rather rapidly from the soil through drainage waters. It is reasonable to suppose that as the lime-charged water percolates through the subsoil, it gradually neutralizes any acids that may be present and thus raises the pH value.

The soil-fertility plots at the New Jersey Agricultural Experiment Station seemed to offer an excellent opportunity for obtaining some definite information on this point. The soil involved is the Sassafras loam and is of fair quality only. It can scarcely be called typical Sassafras soil, since it occurs just at the southern boundary of the soil which is derived from the Triassic red shale. Little is known of the history of this soil previous to 1908, when the plots were laid out and the experimental work was begun. It is quite certain, however, that lime had not been applied for many years and possibly not at all.

During the period of the experimental work certain of these plots have received no commercial lime. Others have been limed regularly at 5-year intervals (carbonate form), some receiving 2 tons to the acre, some 1 ton, and others ½ ton. In all the experiments the land was plowed to a depth as nearly uniform as possible under the circumstances, about 7 inches.

NITROGEN-AVAILABILITY PLOTS

The nitrogen-availability series of plots contains forty $\frac{1}{20}$-acre plots. Half of these have received no lime during the experimental period and the other half have been limed at the rate of 2 tons of the carbonate form to the acre, at 5-year intervals. The last application before the samples under consideration were taken was made in the spring of 1928. Samples of soil were collected from these plots in September 1932. The top or surface sample was taken to a depth of $6\frac{2}{3}$ inches; the second $6\frac{2}{3}$ inches represents the subsoil. The samples were prepared and pH determinations made by the quinhydrone method.

[1] Received for publication Dec. 22, 1933; issued May 1934.

[2] HALL, A. D. THE BOOK OF ROTHAMSTED EXPERIMENTS. Pp. 237–239. London. 1905.

[3] LYON, T. L., and BUCKMAN, H. O. THE NATURE AND PROPERTIES OF SOILS; A COLLEGE TEXT OF EDAPHOLOGY. Rev. ed, p. 290. New York. 1929.

[4] BLAIR, A. W. REPORT OF CHEMIST. Fla. Agr. Expt. Sta. Ann. Rpt. 1911: xxvii–xxxix, illus. 1912.

The results, together with the fertilizer treatments for the plots, are presented in table 1. A study of the table shows that with few exceptions the pH values of the samples from the unlimed plots range from about 5.0 to 5.8, while the pH values of the samples from the limed plots range from a little less than 7 to about 7.5. The subsoils from the unlimed plots are just about as acid as the topsoils. On the other hand, the acidity of the subsoils from the limed plots has been corrected to just about the same extent as the acidity of the surface soils. The figures given in table 1 seem to furnish conclusive proof that the lime, in its journey downward, does neutralize the acidity of the subsoil and thus raise the pH value.

TABLE 1.—*pH values of surface soils and subsoils from nitrogen-availability plots, 1932*

Plot no.	Fertilizer treatment (1/20 acre)	Unlimed section		Limed section	
		Surface soil	Subsoil	Surface soil	Subsoil
		pH	*pH*	*pH*	*pH*
1	None	5.12	5.56	7.55	7.50
2	16 pounds muriate of potash	5.03	5.67	7.45	7.13
3	32 pounds superphosphate	5.09	5.52	7.18	7.15
4	Minerals only [1]	5.23	5.51	7.11	7.22
5	Minerals and 1,600 pounds cow manure	5.78	5.62	7.06	7.10
6	Minerals and 1,600 pounds horse manure (manure discontinued after 1922)	5.32	5.47	6.78	6.68
7	None	4.70	5.30	6.87	7.10
8	Minerals and 8 pounds $NaNO_3$	5.54	5.62	6.88	7.09
9	Minerals and 16 pounds $NaNO_3$	5.50	5.80	7.16	7.20
10	Minerals and $Ca(NO_3)_2$ equivalent to 16 pounds $NaNO_3$	5.49	5.70	7.14	7.30
11	Minerals and $(NH_4)_2SO_4$ equivalent to 16 pounds $NaNO_3$	4.12	4.67	6.00	6.37
12	Minerals and $CaCN_2$ equivalent to 16 pounds $NaNO_3$	5.70	5.58	7.24	7.37
13	Minerals and dried blood (discontinued after 1922)	5.23	5.53	7.05	6.90
14	Minerals, $NaNO_3$, and $(NH_4)_2SO_4$ (half the nitrogen from each)	5.30	5.66	6.90	6.95
15	Minerals and concentrated tankage	5.02	5.27	6.80	6.95
16	Minerals only	5.08	5.06	6.80	7.00
17	——do	5.40	5.46	7.09	6.85
18	Minerals, 1,600 pounds cow manure, and 16 pounds $NaNO_3$	6.06	5.82	7.35	7.32
19	Minerals only	5.15	5.23	7.10	7.28
20	Minerals, 200 pounds wheat straw, and 16 pounds $NaNO_3$	5.80	5.65	7.47	7.45

[1] Minerals = 16 pounds superphosphate and 8 pounds muriate of potash.

CALCIUM AND MAGNESIAN LIMESTONE PLOTS

In a second series of plots representing the same type of soil as the first, magnesian limestone, and high-calcium limestone have been applied over a period of 25 years, at 5-year intervals, in amounts of ½ ton, 1 ton, and 2 tons to the acre. The crops have been largely grain, hay, and forage, though vegetables were grown for a number of years on one section.

Samples of soil were collected from these plots for pH determinations in July 1933. Table 2 shows the pH readings for the surface and subsoils from four different sections that received the lime treatments. An examination of the table shows at once a corresponding rise in the pH value of the surface soil and subsoil as the lime was increased.

TABLE 2.—*pH values of surface soils and subsoils when different lime treatments were applied and different crop rotations grown*

Lime treatment	Rotation 1, plots 21–27, general farm crops		Rotation 2, plots 28–34, general farm crops		Rotation 3, plots 35–41, vegetable crops		Rotation 4, plots 42–48, forage crops and hay	
	Surface soil	Subsoil	Surface soil	Subsoil	Surface soil	Subsoil	Surface soil	Subsoil
	pH	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*
No lime	5.16	5.20	5.48	5.70	5.02	5.17	4.92	5.14
1,000 pounds calcium limestone	5.73	5.70	5.68	6.05	5.10	5.46	5.06	5.58
2,000 pounds calcium limestone	6.07	6.06	6.20	6.36	5.69	6.08	5.75	6.02
4,000 pounds calcium limestone	7.17	7.00	7.20	7.17	6.40	6.92	6.87	7.00
1,000 pounds magnesian limestone	5.78	6.00	6.00	6.05	5.62	5.56	5.73	5.70
2,000 pounds magnesian limestone	6.43	6.54	6.50	6.50	6.20	6.34	6.15	6.16
4,000 pounds magnesian limestone	7.44	7.16	7.46	7.20	7.00	7.22	6.97	7.10

The pH values for the different rotations are generally in fair agreement. For rotations 2, 3, and 4, there is a slight tendency for the pH of the subsoils to be higher than that of the surface soils. Almost without exception the plots that received no lime, both soil and subsoil, have low pH values.

With only one exception the pH values for the magnesian limestone are somewhat higher than those for the corresponding calcium limestone treatments. This is to be expected, since the limestones were used in equal weights rather than in equivalent amounts of actual lime (CaO).

CONTINUOUS WHEAT AND RYE PLOTS

In September 1932 samples of soil were collected for pH determinations from plots on which wheat and rye had been grown continuously since 1909. Lime had been applied usually at 5-year intervals to maintain the reaction at about pH 6.5 to 7. The pH determinations are shown in table 3. The eight samples of surface soil show a range of pH 6.10 to 6.42, and the eight subsoils a range of 5.96 to 6.39. In each case the pH value of the subsoil was only slightly lower than that of the surface soil. A sample of surface soil and subsoil from an adjoining unlimed plot had pH values of 5.5 and 5.38, respectively. In this case the pH value of the subsoil from the limed plot was about 0.8 higher than that of the subsoil from the unlimed plot.

TABLE 3.—*pH values of surface soil and subsoil from plots continuously in wheat and rye, 1909–32*

Plot no.	Crop	pH value of surface soil	pH value of subsoil
68	Rye	6.42	6.39
68–A [1]	do	6.42	6.28
69	Wheat	6.31	6.30
69–A [1]	do	6.31	6.30
70	Rye	6.33	6.20
70–A [1]	do	6.23	6.00
71	Wheat	6.25	6.13
71–A [1]	do	6.10	5.96

[1] The A plots receive each year a top-dressing of nitrate of soda at the rate of 160 pounds to the acre.

LEGUME AND NONLEGUME PLOTS

In series 1 there were eight 1/20-acre plots which had been in a 5-year rotation for 15 years. Lime had been applied at 5-year intervals to maintain the reaction near the neutral point. The crops grown were grain and hay. One half the plots were kept in legumes, when the rotation would permit, and the other half in nonlegumes. Samples of soil were collected from these plots for pH determinations in October 1932. The results are shown in table 4.

TABLE 4.—*pH values of surface soil and subsoil from limed plots in legume and in nonlegume rotations*

SERIES 1

Plot no.	Rotation	pH value of surface soil	pH value of subsoil
73	Nonlegume	7.35	7.20
74	Legume	7.50	7.15
75	Nonlegume	7.38	7.21
76	Legume	7.38	7.12
77	Nonlegume	7.45	7.20
78	Legume	7.55	7.32
79	Nonlegume	7.52	7.30
80	Legume	7.30	7.20

SERIES 2

1	Nonlegume	7.05	7.10
2	do	6.90	6.84
3	Legume	6.48	6.50
4	do	6.72	7.00

With slight exception the eight surface soils show pH values about 0.15 to 0.25 higher than the subsoils. There appears to be no correlation between the soil reaction and the rotation practiced. Samples from unlimed plots adjoining or near the experimental plots gave pH values of about 5.2 to 5.3. Here is a difference of about 2 points between the unlimed surface and subsoils and the corresponding limed soils. It is evident that the lime has influenced the reaction of the soil to a depth of at least 12 to 13 inches.

In series 2 there were 4 plots, 2 of which had been in a nonlegume rotation for about 15 years and 2 in legumes, or legume cover crops, when the rotation would permit. Lime had been applied to keep the soil near the neutral point. Samples of soil were collected for pH determinations in October 1933. The pH values are shown in table 4. On nearby plots without lime the pH value of both surface and subsoil was about 5. It is evident that here, too, the lime had worked downward and changed the reaction of the subsoil to about the same degree as the surface soil.

ALFALFA PLOTS

Four plots had been in alfalfa 12 years during the past 20 years (1914 to 1918, inclusive; 1922; 1925 to 1927, inclusive; 1931 to 1933, inclusive). For the remaining years the crops were corn, oats, wheat, and potatoes. One of these plots had not been limed during the entire 20 years; another had been limed (carbonate form)

at 5-year intervals at the rate of 1,000 pounds per acre; a third had been limed at the rate of 2,000 pounds per acre, and a fourth at the rate of 4,000 pounds per acre. Samples of surface soil and subsoil were collected for pH determinations in July 1933 (table 5).

TABLE 5.—*pH values of the surface soil and subsoil of limed and unlimed plots that had been in alfalfa 12 years during the past 20, and yields of alfalfa hay on the same plots, 1931–33*

Plot no.	Lime treatment (pounds limestone per acre)	Yield of hay per acre				Surface soil, 1933	Subsoil, 1933
		1931	1932	1933	Average		
		Pounds	*Pounds*	*Pounds*	*Pounds*	*pH*	*pH*
1	None	1,713	650	1,861	1,408	5.13	5.24
2	1,000	4,458	2,082	3,943	3,494	5.32	5.31
3	2,000	7,868	3,988	6,925	6,260	5.55	5.55
4	4,000	9,211	5,097	8,087	7,465	6.08	7.05

On the unlimed plot both the surface soil and the subsoil were strongly acid, just above pH 5. On this plot weeds and grasses have practically driven out the alfalfa. On the limed plots the pH values of the subsoil, with the exception of plot 4, were approximately the same as those of the surface soil, and the pH values rose as the lime was increased.

The yields of alfalfa hay for the years 1931 to 1933, inclusive, are shown (table 5) for the purpose of emphasizing the relation between the reaction of the soil and the yield of hay.

SUMMARY

An attempt has been made to determine the extent to which lime, in the carbonate form, applied to the surface soil, works downward and changes the reaction of the subsoil.

The soil used in the tests was a Sassafras loam taken from the soil-fertility plots of the New Jersey Agricultural Experiment Station. Certain of these plots have been limed at 5-year intervals for 25 years; others have received no lime. Samples representing the top 6⅔ inches and corresponding subsoil samples representing the stratum from 6⅔ inches to about 13 inches were collected and the pH values determined.

With few exceptions the figures show that the subsoils from the unlimed plots gave pH readings only a little higher than the corresponding surface soils. In most cases there was very little difference between the pH values of the surface and subsoil from the limed plots. Both the surface and subsoil of these plots gave pH readings about 1½ to 2 points higher than those of the corresponding unlimed plots.

It is thus established that in the case of this particular soil carbonate of lime used at the rate of 2,000 to 4,000 pounds to the acre over a period of 25 years has changed the reaction of the subsoil (6⅔ to 13 inches) to about the same degree that it has changed the reaction of the surface soil. It seems reasonable to conclude, therefore, that in general where generous applications of lime are made on acid soils, the acidity of the subsoil will also be gradually corrected.

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